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Effect of Cobalt in Diet of the Chick.* (20058)

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It has been well established that dietary cobalt will stimulate the growth of ruminants if the diet is low or lacking in vit. B_{12}. Research conducted(1,2) since its chemical isolation has shown that vit. B_{12} contains cobalt. Abelson and Darby(3) showed that dietary cobalt was used by ruminants in the synthesis of vit. B_{12} presumably by the action of the rumen microflora. Becker *et al.*(4) have shown that cobalt might possibly have other uses by sheep beyond that used directly for vit. B_{12} synthesis. A later report by Smith *et al.*(5) showed that these early results reported by Becker *et al.*(4) occurred because the amounts of vit. B_{12} used were too small. Injections of 150 μg or more of vit. B_{12} overcame cobalt deficiency symptoms. Davis and Chow(6) used 2 different levels of radioactive Co^{60} in diets for rats and obtained a proportionate increase in the radioactive and microbiological vit. B_{12} content of the feces. These increases appeared to be greatly accelerated by the addition of aureomycin to the diet. When Cuthbertson *et al.*(7) fed Co^{60} to rats over long periods of time in a vit. B_{12} deficient diet they were unable to isolate tagged vit. B_{12} from the livers. It is interesting to note that the amount of Co^{60} fed was small and

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that there was no antibiotic included in the diet.

In the studies of Tappan and coworkers(8) the growth of rats was not significantly affected by the feeding of 0.1 mg per day of cobalt in a corn-soybean oil meal diet containing vit. B_{12}. When cobalt was fed at the level of 1.0 mg per day growth was depressed. Klosterman *et al.*(9) have obtained faster growth with swine fed a diet deficient in vit. B_{12} by supplementing this diet with a trace-mineralized, iodized salt containing 0.026% cobalt. Very little work has been done with cobalt in the vit. B_{12} deficient chick. Halbrook *et al.*(10) obtained slightly increased chick growth when cobalt chloride was added to unchanged litter at the rate of 2 g per square foot. When added to the basal diet at the rate of 3½ g of cobalt chloride per 100 lb of ration no growth stimulation occurred. Briggs *et al.*(11) reported that a low level of cobalt (1 mg/kg) was ineffective in promoting growth in a vit. B_{12} deficient diet.

The purpose of the studies reported here was to determine whether dietary cobalt would affect the growth of the chick if fed diets containing an antibiotic compounded with and without vit. B_{12}.

Materials and methods. Day-old White Leghorn female chicks from the Poultry Division flock were used in groups of 10. They were sexed shortly after hatching and reared in electrically heated metal batteries with raised wire screen floors. Feed was supplied *ad libitum* and the water changed daily. The basal ration consisted of the following ingredients (g/100.23 g): ground yellow corn, 61; solvent process soybean oil meal, 35; dicalcium phosphate, 1.5; iodized sodium chloride, 1; manganese sulfate, 0.03; chick size oyster shell, 1.5; and 3000A-400D fish oil, 0.2. In addition 0.33 mg of crystalline riboflavin and 2.5 mg of procaine penicillin G were included. 1.5 μg of crystalline vit. B_{12} was added to the diet of some of the groups as indicated in the tables. The cobalt used was supplied in the form of $CoSO_4 \cdot 7H_2O$ which contained less than 0.4% impurities. This compound is 20.9% cobalt.

Results and discussion. The addition of cobalt sulfate to the diet did not significantly affect chick growth, feed efficiency, or death loss when the diet contained vit. B_{12} (Table I). High levels of cobalt had no observable toxic effect on the chicks. These results indicate that the chick might have a much greater tolerance for dietary cobalt than the rat(8) although the penicillin included in these diets might have had an effect on this tolerance.

TABLE I. Effect of Cobalt on Chicks Fed Diets Containing Vitamin B_{12}.

Trial	Group	$CoSO_4 \cdot 7H_2O$ /kg of basal ration, mg	Avg wt, g	No. chicks dead	Feed efficiency*
1†	1	0	415	1	2.56
	2	6	389	0	2.44
	3	10	401	0	2.49
	4	14	368	0	2.67
	5	50	394	0	2.52
2‡	1	0	341	1	2.47
	2	25	339	0	2.47
	3	50	340	1	2.45
	4	100	350	0	2.42
	5	150	347	0	2.24
	6	200	335	0	2.36
	7	300	355	1	2.30

* Feed efficiency $= \frac{\text{Total feed consumed}}{\text{Wt gained}}$.

† Carried on exp. 37 days.
‡ " " " 34 " .

The results given in Table II show that growth stimulation occurred when 10 to 100 mg of cobalt sulfate were added to each kg of the vit. B_{12} deficient diet. Although analysis of variation due to treatment does not show significance below the 5% level, the authors feel that the differences due to cobalt feeding are large enough to show nutritional importance. Because of the relatively high levels of cobalt sulfate which were necessary to produce the apparent growth stimulation it is felt that this effect might be caused by the stimulation or alteration of the intestinal flora to synthesize vit. B_{12}. In spite of the fact that all the chicks were raised on wire screen floors, vit. B_{12} present in the voided feces (or synthesized after voiding) and possibly consumed by the chicken may have been a factor in these results. Also, the cobalt content of the basal diet, present as an impurity, and the presence of an antibiotic may have been factors in-

TABLE II. Effect of Cobalt on Chicks Fed Diets Deficient in Vitamin B_{12}.

Trial	Group	$CoSO_4 \cdot 7H_2O$ /kg of basal ration, mg	Avg wt, g	No. chicks dead	Feed efficiency*
1†	1	0	309	1	2.93
	2	6	308	0	3.01
	3	10	336	1	2.90
	4	14	345	1	2.73
	5	50	356	1	2.91
	6	Pos. control (with B_{12})	415	1	2.56
2‡	1	0	246	1	2.50
	2	10	260	0	2.53
	3	50	279	1	2.83
	4	100	298	1	2.60
	5	Pos. control (with B_{12})	294	0	2.52

* Feed efficiency $= \frac{\text{Total feed consumed}}{\text{Wt gained}}$.

† Carried on exp. 37 days.
‡ " " " 35 " .

volved in this stimulatory effect (the cobalt content of the ration and of the water consumed, was not determined, unfortunately).

Jaffé(12) reported a beneficial action of cobalt on pregnancy and lactation of rats and mice deficient in vit. B_{12}. It appears possible, therefore, that under certain conditions cobalt may improve the nutrition of vit. B_{12}-deficient, non-ruminating animals.

Summary. 1. Chick growth was stimulated by the addition of 10 to 100 mg of cobalt sulfate to each kg of a corn-soybean oil meal diet which was deficient in vit. B_{12}. 2. When similar levels of cobalt sulfate were added to the same diet supplemented with ample vit. B_{12} they produced no observable effect.

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Inhibition of Rat Liver Succinoxidase by Testosterone-17β Diethyl Aminoethyl Carbonate.* (20059)

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Various natural and synthetic estrogens exert an *in vitro* inhibitory action on the succinoxidase activity of many tissues(1). Utilizing synthetic estrogens, McShan and Meyer(2) have localized this inhibition to the cytochrome oxidase component of the system while Case and Dickens(3) demonstrated that, depending upon the estrogen used, the point of action could be succinic dehydrogenase, cytochrome oxidase, or a hypothetical link between these two enzymes. Guidry *et al.*(4) found, however, that estrone and estradiol did not inhibit the oxidation of succinate by rat liver homogenates. Androgens inhibit the oxygen consumption of rat liver,

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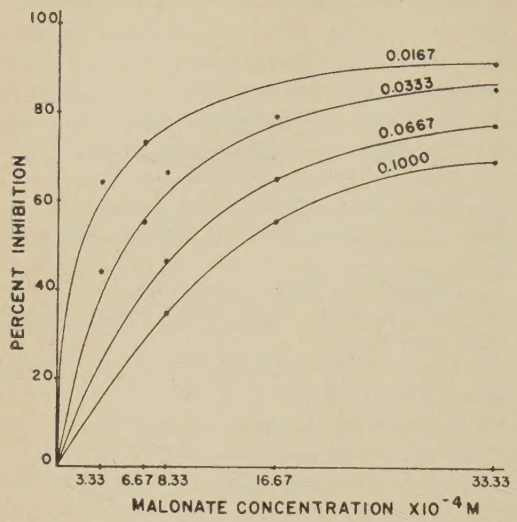

FIG. 1. % inhibition of succinoxidase plotted against malonate concentration. Final molarity of sodium succinate indicated on each curve.

kidney, and brain slices(5), rat brain homogenates(6), and rat striated muscle(7), but the mechanism or significance of these inhibitions is not clearly understood. Kalman(8) has shown that the inhibition of rat liver succinoxidase by testosterone-17β diethyl aminoethyl carbonate hydrochloride, a water-soluble compound of weak androgenic activity, might be by way of succinic dehydrogenase.

In the present report, the nature of the *in vitro* inhibition of rat liver succinoxidase by testosterone-17β diethyl aminoethyl carbonate† is further elucidated.

Materials and methods. Adult Sprague-Dawley rats were killed by decapitation and 5% liver homogenates were made up in ice-cold distilled water using an all-glass homogenizer. The reaction mixture was prepared in Warburg flasks as described by Umbreit *et al.* (9), and contained 1.0 ml of 0.1 M phosphate buffer (pH 7.4), 0.2 ml of 5% homogenate, 0.3 ml of 4×10^{-3} M $CaCl_2 \cdot 2H_2O$, 0.3 ml of 4×10^{-3} M $AlCl_3 \cdot 6H_2O$, 0.4 ml of 10^{-4} M cytochrome C (standardized colorimetrically according to the method described by Umbreit *et al.*(9)), sodium succinate in varying concentrations, inhibitors in varying concentrations, and sufficient distilled water to make 3 ml. The center wells contained 0.2 ml of 10% KOH. After introduction into the bath (37°C), the flasks were gassed with 100% O_2 for 10 minutes and manometer

† This compound was generously contributed by Dr. Ernst Oppenheimer of the Ciba Corp.

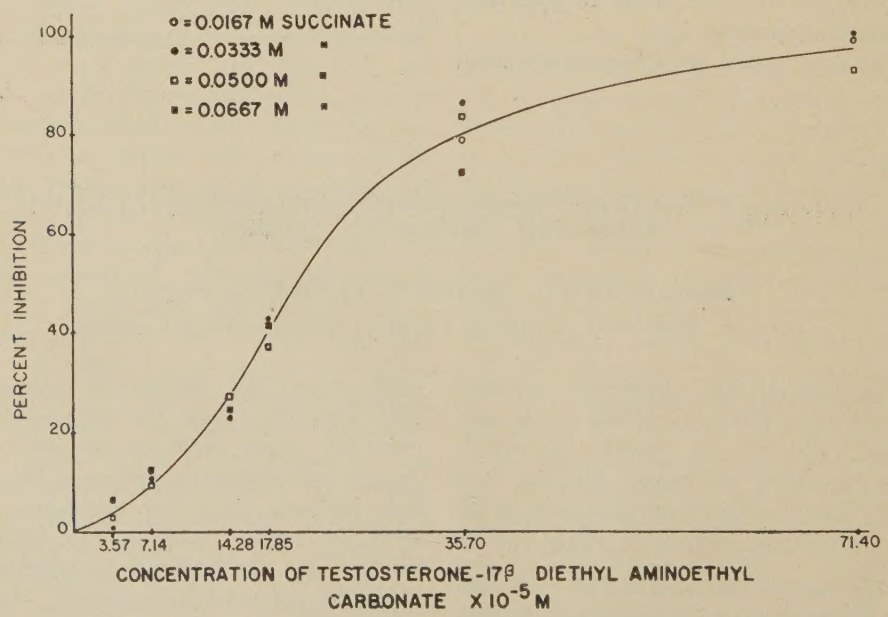

FIG. 2. % inhibition of succinoxidase plotted against concentration of testosterone-17β diethyl aminoethyl carbonate.

TABLE I. Fractional Activity of Rat Liver Succinoxidase in the Presence of Various Concentrations of Testosterone-17β Diethyl Aminoethyl Carbonate.

Final succinate conc.	v_i at indicated final molarity of testosterone-17β diethyl aminoethyl carbonate			
	3.57×10^{-5}	7.14×10^{-5}	14.28×10^{-5}	17.85×10^{-5}
.0333 M	100	89.6	77.3	57.7
.0500	97.1	89.6	72.8	62.7
.0667	93.5	87.6	75.7	57.7

v_i is expressed as % of non-inhibited succinoxidase activity.

readings were taken every 10 minutes for the next 40 minutes. The inhibitors used were sodium malonate and testosterone-17β diethyl aminoethyl carbonate. The former is a known competitive inhibitor of succinic dehydrogenase(10) and was employed in the present studies for comparison with the inhibitory effects of the androgen. The androgen was received as the hydrochloride and preliminary experiments indicated that it was sufficiently acid, even in very low concentrations, to cause extensive tissue damage. Therefore, it was titrated with dilute NaOH to a point at which it was barely soluble before addition to the flasks.

Results. In Fig. 1-2 all experiments are summarized graphically by plotting % inhibition against concentration of inhibitor at several levels of substrate. With malonate as the inhibitor (Fig. 1), the degree of inhibition is dependent upon substrate concentration, indicating competitive inhibition. In the presence of the androgen (Fig. 2), the degree of inhibition is independent of substrate concentration, suggesting non-competitive inhibition. When the 1/v:1/s plot described by Lineweaver and Burk(11) is applied to the same data, confirmation is obtained for the competitive inhibition with malonate and the non-competitive inhibition with testosterone-17β diethyl aminoethyl carbonate.

Representative data are presented in Table I and treated in Fig. 3 according to the method described by Ebersole *et al.*(12). In plotting v/v_i:(I) (v = reaction velocity in absence of inhibitor, v_i = reaction velocity in presence of inhibitor, and (I) = concentration of inhibitor) for several concentrations of substrate, competitive inhibition is demonstrated by a series of straight lines with unit intercept on the ordinate and with slope dependent upon substrate concentration. The malonate-inhibited system satisfies these conditions. Non-competitive inhibition is demonstrated when the plots form lines that coincide (Fig. 3), indicating that slope is independent of substrate concentration. The lines will not coincide if the substrate concentration is limiting.

The plots in Fig. 3 were derived from the equation, $v/v_i = 1 + (I)/K_i$, representative of a straight line. Since the graph in Fig. 3 is concave upward it will not satisfy this equation. The appropriate form then becomes $v/v_i = 1 + (I)^r/K_i$, in which r equals the number of molecules of inhibitor which combine with one molecule of enzyme(12). By plotting log $(v/v_i - 1)$:log(I), a straight line is obtained with slope equal to r(12). This procedure is carried out in Fig. 4 and it is found that r equals 2.

Discussion. While many estrogenic compounds act as enzyme inhibitors *in vitro* they do not appear to do so *in vivo*(1). It seems, therefore, that the hormones may possess chemical properties which, while readily de-

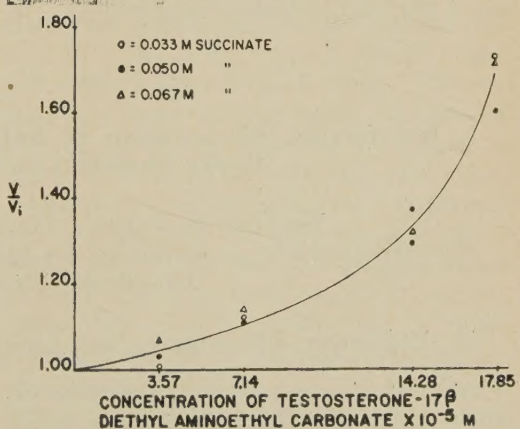

FIG. 3. Alternative plot demonstrating non-competitive inhibition of rat liver succinoxidase by testosterone-17β diethyl aminoethyl carbonate. Method described by Ebersole *et al.*(12).

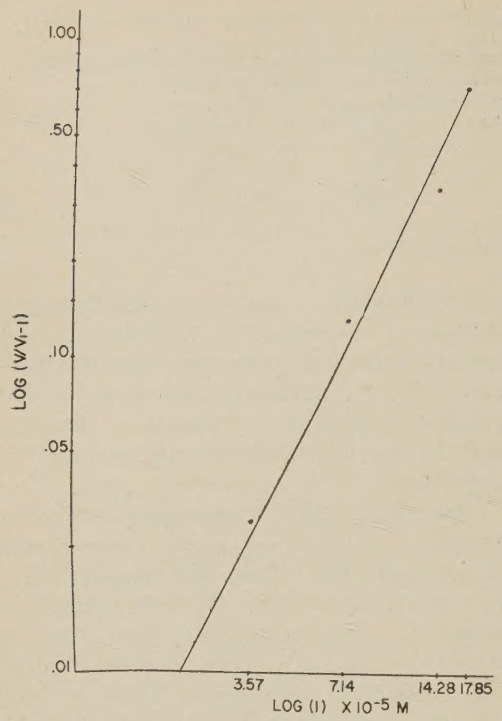

FIG. 4. Double log plot showing slope of line equal to 2. Method described by Ebersole *et al.* (12).

tectable *in vitro,* have little or nothing to do with their physiological effects when injected. The androgens have not been as thoroughly studied as the estrogens in this regard. The inhibitory property of testosterone-17β diethyl aminoethyl carbonate might reside, for the most part, in the C-17 substituted grouping, as has been previously suggested(5). In spite of this, the compound was used in the present study because of the technical advantage of its water-solubility.

Summary. It has been demonstrated that the *in vitro* inhibition of rat liver succinoxidase activity by testosterone-17β diethyl aminoethyl carbonate is non-competitive, with 2 molecules of inhibitor apparently interacting with one molecule of enzyme.

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Interference Phenomenon in Animal Infections with Rickettsiae of Rocky Mountain Spotted Fever.* (20060)

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The interference phenomenon has been

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I wish to thank Dr. Carl Larson, director of the Rocky Mountain Laboratory, National Microbiological Institute, National Institutes of Health, Hamilton, Montana, for placing the facilities of this laboratory at my disposal. Many of the experiments were carried out at this laboratory.

† The writer was assisted in these experiments by Mr. C. H. Sawyer, Mr. R. Layton, and Miss Hope Emerson.

demonstrated for many animal, plant, and bacterial viruses. Although they present endless variety and detail, the general characteristics of these phenomena are well established. While there have been no reports of interference between rickettsiae, it seemed possible that these agents might also be subject to infection interference, since rickettsiae are also intracellular parasites. In the course of studies on the epidemiology of Rocky Mountain spotted fever, the experiments which are here reported were undertaken to test the validity of this hypothesis.

Materials and methods. The two strains of *R. rickettsii* employed in these experiments have been previously described(1). Strain T is of low virulence, rarely causing scrotal reactions and is not fatal for guinea pigs. The R strain, on the other hand, causes a marked scrotal reaction resulting in necrosis, and kills some of the inoculated guinea pigs. Strains R and T are similar in that both possess toxin, a hemolytic factor, and are practically immunologically identical(1). Material for inoculation was obtained by egg passage, using chick embryo yolk sacs. All egg titrations to determine the LD_{50} of the various suspensions were carried out in 5-day-old chick embryos. 0.5 ml of the various suspensions was inoculated into the yolk sac and incubated at 35°C. Eggs dying within 48 hours were discarded. The remaining eggs were examined daily for 12 days. Ten to 12 eggs were used per dilution. All dilutions were carried out in Snyder's solution(2). The strains of other rickettsiae employed were those being carried at the Rocky Mountain Laboratory (U.S.P.H.S.), Hamilton, Montana. The Nine-Mile Q fever strain, a Malayan mite strain of scrub typhus, the Brienl strain of epidemic typhus, a strain of Boutonneuse fever, and the Wilmington strain of endemic typhus were employed in the various experiments. Egg material was used for inoculation for all of the above strains except in the case of scrub typhus, where mouse spleen was used. All guinea pigs used were healthy male animals weighing between 350-450 g. Of the wild animals, the Columbian ground squirrels, field mice (Microtus), and cottontail rabbits were trapped in the Bitterroot Valley, Montana. None of the animals employed showed any complement-fixing antibodies against any of the spotted fever strains used in these studies. The *D. andersoni* nymphs used in all experiments had been infected as larvae and nymphs with either the T or the R strain by feeding on infected guinea pigs. After the female laid eggs, the resulting larvae were infected with the appropriate strain and allowed to molt to nymphs. The above procedure was repeated once more.

TABLE I. Interference between Infection with a Strain of Low and One of High Virulence of *R. rickettsii*, as Demonstrated by Injection into 5 Series of 10 Guinea Pigs Each.

Strain injected	Fatality	Avg days of fever	4+ scrotal reaction
T	* 0/50	5.1 ± .92	† 0/50
R	14/50	8.1 ±1.2	45/50
T + R	0/50	5.3 ± .82	6/50

* Numerator shows No. of animals dying from spotted fever.

† Numerator shows No. developing severe scrotal reactions. Four animals inj. with T strain followed by R strain developed a slight scrotal reaction leading to swelling and redness which disappeared after a few days.

Results. Two series of experiments were performed. The first series of experiments shown in Table I were carried out as follows: 3×10^5 LD_{50} (determined by egg titration) of the weakly virulent T strain was injected intraperitoneally into 10 male guinea pigs. Four hours later, 10^4 LD_{50} (determined by egg titration) of the virulent R strain was injected intraperitoneally into the above animals. Ten control animals were injected with 3×10^5 LD_{50} of the T strain, and 10 guinea pigs with 10^4 LD_{50} of the R strain. The animals were observed for 2 weeks. A temperature of 39.8°C or over was considered as fever. The results are cumulative data from 5 experiments, all giving the same qualitative and approximately the same quantitative results. 4+ scrotal reactions indicate severe reaction leading to hemorrhage and necrosis. From Table I it is obvious that a weakly virulent strain of *R. rickettsii* protected animals against a virulent strain of the disease. Other experiments showed that under conditions similar to those shown in Table I, strain T (a) protected animals when both T and R were given simultaneously, or when R was

injected anywhere up to 14 days after the T strain (cf. footnote‖), (b) protected about 40% of the guinea pigs when only 3×10^4 LD_{50} of strain T was injected, (c) interfered with the R strain when inactivated by ultraviolet irradiation but not by heat, and (d) protected animals against boutonneuse fever.

In other experiments involving quantitative centrifugation and highly purified rickettsiae (1) the results indicated that the protective property is a part of the rickettsiae and not some other substance present in the infected yolk sacs.

Suspensions of rickettsiae causing Q fever, scrub typhus, epidemic typhus, and endemic typhus, which are immunologically unrelated to *R. rickettsii,* were substituted for Rocky Mountain spotted fever strain T. Under the conditions shown in Table I, suspensions of these immunologically distinct rickettsial strains also protected about the same percentage of animals against injections of the virulent R strain. The animals did develop the typical symptoms caused by the rickettsiae used to protect against spotted fever. Not only did these rickettsiae protect animals against the symptoms of Rocky Mountain spotted fever, but in guinea pigs inoculated with *C. burneti* (Q fever) followed 4 hours later by an injection of *R. rickettsii,* there was 1000 times less the maximum number of Rocky Mountain spotted fever organisms in the testis-tunica than was found in the control animals, which received an injection of strain R alone. A typical experiment is shown in Table II. Twelve male guinea pigs were injected intraperitoneally with 1×10^6 LD_{50} (determined by egg titration) of *C. burneti.* Four hours later they were injected intraperitoneally with 1×10^4 LD_{50} of strain R of *R. rickettsii.* Twelve control guinea pigs were injected intraperitoneally with 1×10^4 LD_{50} of strain R. At 8, 72, 120, and 230 hours following inoculation of strain R, the testes-tunicas were taken from 2 pigs from each group and combined, and 10% suspensions prepared in Snyder's solution(2). Each suspension was titered in guinea pigs which had recovered from an infection of *C. burneti.* These animals had been injected with 1×10^5 LD_{50} of *C. burneti* 2 months previously. All these guinea pigs showed complement-fixing antibodies of over 1:512. Five of these animals were used per dilution to titrate each testis-tunica suspension. The control series were also titrated in the Q fever immune animals. Control experiments showed that the testis-tunica suspensions from the animals injected with *C. burneti* and *R. rickettsii* contained no demonstrable inhibitors, which would prevent the suspensions from showing higher spotted fever titers if larger numbers of strain R of *R. rickettsii* had been present.

TABLE II. Multiplication of *R. rickettsii* in Testes-Tunicas of Guinea Pigs Injected 4 Hours Previously with *C. burneti.*

Time after *R. rickettsii* inj. (hr)	Min. infectious doses (ID_{50}) in testis-tunica suspensions	
	Q + R	R
8	0	0
72	10	1000
120	1	10000
230	0	100

The second series of experiments shown in Table III were carried out as follows: 100 *D. andersoni* nymphs infected with the low virulent strain T and 2 *D. andersoni* nymphs infected with the highly virulent strain R were put on each of 3 Columbian ground squirrels. After 3 days at which time 80% of the nymphs infected with the T strain and both nymphs infected with the R strain had been feeding, 100 nymphs which had been raised through many generations in the laboratory and were free of spotted fever, were put on each of the 2 squirrels. The uninfected nymphs were allowed to engorge and molt to adults. After one month at room temperature, 30 nymphs from each squirrel were put on each of 2 guinea pigs. The nymphs were allowed to feed for 6 days and the animals then observed for 3 weeks. In control experiments, the lowly virulent and highly virulent infected samples (Table III, 1 and 2) were set up exactly as described above except that sample 1 had no highly virulent infected ticks and sample 2 no low virulent infected ticks. The results are the cumulative data from 4 experiments, all experiments giving the same results. It can be readily seen that uninfected ticks feeding on Columbian ground squirrels harboring a large number of *D. andersoni* in-

TABLE III. Interference between Infections with a Strain of Low and One of High Virulence of *R. rickettsii* as Demonstrated by Feeding *D. andersoni* Ticks on Ground Squirrels.

Normal ticks fed on squirrels infected by exposure to		Reactions of guinea pigs on which normal ticks fed after feeding on squirrels		
		Avg days of fever	Scrotal reaction	Fatality
Low virulent infected ticks	(1)	5.1 ± .93	* 0/20	†0/20
Highly	(2)	8.2 ±1.4	17/21	8/21
Low and highly	(3)	4.6 ± .81	0/19	0/19

* Numerator shows No. of animals showing scrotal reaction. Four animals in group (1) showed no symptoms, 3 animals in group (2) showed no symptoms, and 5 animals of group (3) showed no symptoms. Fever equals any temperature over 39.8°C. All scrotal reactions were severe resulting in necrosis in group (2).

† Numerator shows No. of animals dying of spotted fever.

fected with a low virulent strain and a small number of *D. andersoni* infected with a highly virulent strain of *R. rickettsii,* transmit only a low virulent strain to animals. It is not possible to say from these experiments whether interference between the high and low virulent strains occurs in the ticks. All tests carried out so far indicate that such ticks contain only the low virulent strain. Larvae coming from female of such ticks have been found to have the same strain as the parent. Experiments similar to the above were carried out with field mice (Microtus) and cottontail rabbits with similar results. In other experiments, in which ticks harboring highly virulent strains predominated, "normal" ticks became infected only with the highly virulent strain. Larvae coming from females of such ticks have been found to have the same strain as the parent.

Discussion. The current hypothesis is that interference between viruses is due to competition for susceptible cells. This idea is based mainly on the work carried out with bacteriophage and the influenza-chick embryo system.

In the experiments described in this paper, in which various strains of rickettsiae protect guinea pigs against virulent spotted fever, there are many similarities to the interference phenomena reported for viruses. However, two lines of evidence indicate that the protection against virulent spotted fever may not be due to competition for susceptible cells. In the first place, only 10^7 to 10^8 rickettsiae‡ per animal were injected to establish protection. Taking into account that spotted fever is a rather generalized infection it does not seem likely that anywhere near all the susceptible cells are invaded. Indeed, direct evidence supporting this contention has recently been found. Secondly, 15§ days after the animals are injected with Q fever rickettsiae, which are immunologically distinct from *R. rickettsii,* the animals are no longer protected against an injection of virulent spotted fever,‖ although at this time all the organs show many more cells infected with Q fever rickettsiae than were present in the initial 4 hour post-inoculation time, during which time the animals were protected against an injection of virulent *R. rickettsii.* Thus, while there are similarities between the experiments described in this paper and those involving virus interference, future experi-

‡ The rickettsiae were counted by the electron microscope method described previously(1).

§ Animals injected with Q fever are resistant to the R strain to about 10 days post-inoculation. That is to say, animals injected with Q fever are not resistant to an injection of spotted fever given about 2 weeks later. At no time during the course of infection in Q fever infected animals, including first to thirtieth day post-inoculation period, does sera of such animals give any antibody reaction against R strain as measured by complement fixation activity, neutralization of spotted fever toxin in mice.

‖ This does not hold for the low and highly virulent strains of spotted fever since immunologically they are practically identical, and by 15 days antibodies begin to appear in blood of the animal. From this time on, therefore, protection given by the low virulent strain against virulent strain may be due entirely to immune response.

ments must decide whether the "interference phenomenon" observed in the two classes of infectious agents is due to the same mechanism.

The "interference phenomenon" reported in this paper involving *D. andersoni* and small wild animals may have important implications in explaining the epidemiology of Rocky Mountain spotted fever. It may be involved in the explanation of the contention that in certain limited localities strains having high and low virulence persist year after year(3). Since a similar situation is found in scrub typhus(4), it is possible that such an "interference phenomenon" is important in the epidemiology of this rickettsial disease. However, much more work is necessary before final conclusions can be drawn, since many factors would be involved in determining the importance of such a phenomenon in nature.

It should be mentioned that with certain strains of lower virulence than the T strain, it has not been possible to infect cottontail rabbits, Columbian ground squirrels, field mice, or chipmunks, so that such animals will infect *D. andersoni*. These animals are among the main animal reservoirs for larvae and nymphs of *D. andersoni*. Multiplication of the rickettsiae of these strains of very low virulence could also not be demonstrated by repeated egg titrations of the organs and blood of the above animals. On the other hand, these strains grow about as well in *D. andersoni* and chick embryos as the virulent strains. It is possible that they are maintained by hereditary transmission. These low virulent strains, however, will still protect animals against highly virulent strains under the conditions described in Table I. The elucidation of the mechanism by which these strains are maintained in nature is of importance in understanding the epidemiology of not only Rocky Mountain spotted fever, but perhaps also of other rickettsial diseases.

Summary. 1. Eighty to 90% of the guinea pigs infected intraperitoneally with a strain of low virulence of *R. rickettsii* were protected against a simultaneous injection of a highly virulent strain of spotted fever, or boutonneuse fever, provided the low virulent strain was given in about 10 to 30 times the concentration of the virulent strain. 2. Infection with rickettsiae of Q fever, scrub typhus, endemic typhus, and epidemic typhus protected guinea pigs against a virulent strain of spotted fever under the above conditions. 3. Columbian ground squirrels, field mice (Microtus), and cottontail rabbits were infected by exposure to many *D. andersoni* containing a low virulent strain of Rocky Mountain spotted fever and a few *D. andersoni* containing a highly virulent strain. Uninfected ticks fed upon these animals were found to contain only the low virulent strain.

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Role of Thioctic Acid in the Transfer of Acyl Groups.* (20061)

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Preliminary data(1) have suggested that in the oxidation of pyruvate thioctic acid has no role in the transfer of electrons over DPN,†

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† The following abbreviations are used: Co A, coenzyme A; $DPN_{ox.}$ and $DPN_{red.}$, oxidized and reduced diphosphopyridine nucleotide; ATP, adenosine triphosphate.

but rather is essential for acetylation of Co A. The present investigation was undertaken to study in more detail the influence of thioctic acid in pyruvate oxidation, and in the analogous oxidation of α-ketoglutaric acid(2,3).

Methods. Pure cultures of the ciliate *Tetrahymena pyriformis* S‡ were grown in 5 gallon pyrex carboys containing 10 liters of 0.5% bacteriological peptone, 0.1% yeast extract and 8 p.p.m. of Dow Corning Anti-foam A. The carboys were well aerated on a vacuum line at room temperature (24-28°C). After 72 hours of growth the cells were harvested with the aid of a Sharples supercentrifuge and were washed 3 times with 5 volumes of distilled water. The washed cell paste was ground with ½ weight of powdered quartz in a chilled mortar and the mixture was brought into solution at pH 7.4. The quartz was removed by gentle centrifugation and the supernatant fractionated by isoelectric precipitation according to the procedure of Green *et al.*(5). The fraction which precipitated at pH 5.4 showed high pyruvic and α-ketoglutaric oxidase activity. In early experiments(1), the preparations were heated for 10 min at 54°C to destroy phosphotransacetylase activity. Later it was found by testing in the arsenolysis reaction(6) that *Tetrahymena* does not contain this enzyme. This is consistent with the previous observation that acetyl ~ phosphate is not formed in the course of acetate metabolism by this organism(7). However, heating coagulates many inactive proteins and enzymes which are present in the preparation after isoelectric precipitation. Therefore, this procedure was retained as the final step in the purification. Sodium pyruvate, α-ketoglutarate, ATP, and cocarboxylase were obtained commercially. Samples of highly purified Co A were obtained from Dr. F. M. Strong and from the Pabst Laboratories. Thanks are also extended to Dr. Helmut Beinert for a supply of acetyl ~ Co A. Thioctic acid was generously provided by Dr. E. L. R. Stokstad. *Oxidative activity* of pyruvic and α-ketoglutaric oxidase was measured spectrophotometrically following the rate of reduction of the dye, 2,6-dichlorophenolindophenol at 600 mμ. Optical density (O.D.) readings were taken at intervals for 3 min following addition of the enzyme and the values plotted against time. Oxidative activity is expressed as the decrease in O.D./min. A decrease in O.D. of 0.01/min corresponds approximately to the oxidation of 0.1 μM of substrate/hr. *Acyl groups* were measured by the hydroxamic acid method(8) and sulfanilamide by the colorimetric method of Bratton and Marshall(9). Acetate was determined enzymatically(10). Diacetyl and acetoin were determined by the method described by White *et al.*(11).

‡ This organism had previously been classified as *T. geleii* S. However Corliss(4) has indicated that *T. pyriformis* S conforms with correct nomenclature.

TABLE I.

Reaction mixture	Indophenol reduction/min. Pyruvic oxidase, O.D.	α-ketoglutaric oxidase, O.D.
Complete system	.057	.048
No ATP	.059	.052
" cocarboxylase	.048	.050
" DPN	.053	.057
" Co A	.051	.053
" $MgCl_2$	.002	.003
" substrate	.006	.002

Requirements for oxidative activity of pyruvic oxidase and of α-ketoglutaric oxidase. The complete system contained 20 μM of $MgCl_2$, 200 μg cocarboxylase, 10 μM ATP, 50 μg DPN, 60 units of Co A, 200 μM phosphate (pH 6.8), 20 μM of pyruvate or α-ketoglutarate, .10 ml of enzyme for pyruvic oxidase or .13 ml for α-ketoglutaric oxidase, and 2,6-dichlorophenolindophenol in 3 ml. The initial optical density was .410.

TABLE II.

Reaction mixture	Acylhydroxamic acid formed. Pyruvic oxidase, μM	α-ketoglutaric oxidase, μM
Complete system	.53	.58
No ATP	.58	.72
" cocarboxylase	.72	.64
" DPN	.64	.67
" Co A	.08	.06
" $MgCl_2$	.02	.09
" cysteine	.06	.06
" substrate	.04	.07

Requirements for acylation component of pyruvic oxidase and of α-ketoglutaric oxidase. The complete system contained 70 μg of cocarboxylase, 10 μM of ATP, 20 units Co A, 10 μM $MgCl_2$, 100 μg DPN, 10 μM of cysteine, 100 μM $NaHCO_3$, 10 μM of pyruvate or α-ketoglutarate, 3 μM hydroxylamine hydrochloride, .20 ml enzyme for pyruvate oxidation or .26 ml for α-ketoglutaric oxidation in 1 ml. Incubated 60 min. at 25°C.

TABLE III.

Enzyme treatment	Indophenol reduction/min.		Acylhydroxamic acid formed	
	Pyruvic oxidase, O.D.	α-ketoglutaric oxidase, O.D.	Pyruvic oxidase, μM	α-ketoglutaric oxidase, μM
0	.053	.051	.63	.58
0 + 20 units/ml thioctic acid	.052	.050	.59	.61
Alumina	.048	.042	.03	.06
Alumina + 20 units/ml thioctic acid	.054	.048	.61	.64

Effect of thioctic acid on acyl formation and on oxidative activity. The incubation mixtures were as indicated in the legends of Tables I and II except that ATP was omitted from both incubation mixtures and that Co A was omitted from the oxidative mixture.

Pyruvate was estimated as the dinitrophenylhydrazone(12) and acetaldehyde was determined according to Stotz(13). Ferricyanide was measured by treating samples with $ZnSO_4$, HCl, and KI, and then titrating the liberated iodine with dilute thiosulfate(14).

Results. The enzyme which precipitates between pH 5.4 and 6.3 contains high activity for the acylative and oxidative components of pyruvic and α-ketoglutaric oxidases. The activities of the two oxidases, however, are not identical. It was found that activity toward α-ketoglutarate was only approximately 77% of that toward pyruvate. In order to better compare the properties of the two oxidations, the amount of enzyme added to incubation mixtures when α-ketoglutaric oxidase was studied was therefore increased to 1.3 times the amount used when pyruvic oxidase was examined.

Neither cocarboxylase, DPN, nor ATP are necessary for optimal activity of either component of the respective oxidations (Table I). Sufficient cocarboxylase and hydrogen acceptor, which would be expected to be requirements of the system, are apparently retained in the enzyme preparation. Similar non-requirement for these cofactors has been found in studies with partially purified mammalian oxidases(2,5). Cocarboxylase and DPN were nonetheless routinely added to incubation mixtures containing these enzymes. The acylative and oxidative steps of both enzymes have absolute requirements for Mg^{++}. In addition, Co A and cysteine are essential for acylative activity of both pyruvic and α-ketoglutaric oxidases (Table II).

The *Tetrahymena* preparation contains rather high amounts of thioctic acid. This factor however can be readily removed by merely adding a small amount of alumina.§ The mixture is then gently shaken for a few seconds, and the alumina removed by gentle centrifugation. The supernatant is free of thioctic acid when assayed for acetate replacing ability in the growth of *Lactobacillus casei*(15) and as a cofactor for acylative reactions carried out by living *Tetrahymena*. The *Tetrahymena* assay detects total protogen, bound as well as free forms. Whereas the rate of oxidation of pyruvate and α-ketoglutarate by the supernatant remains unchanged following alumina treatment, such thioctic acid-free preparations do not form acyl groups in the course of the respective oxidations (Table III). The addition of pure thioctic acid completely restores acylative activity to the level of activity prior to alumina treatment. The optimal concentration of thioctic acid required for acylative activity is in the region of 20 units/ml (Fig. 1). Addition of thioctic acid in amounts of 20 units/ml, to both untreated and alumina treated enzyme did not significantly alter the rate of dye reduction when either pyruvate or α-ketoglutarate served as substrate.

The effect of thioctic acid on the acetylation of sulfanilamide from acetyl ~ Co A was studied to ascertain whether the transfer of acyl groups *from* Co A as well as the transfer *to* Co A is dependent upon the factor. Table IV shows that alumina treatment of duck liver acetylase (prepared by the method of Kaplan and Lipmann)(16) is without effect on the rate of acetylation of sulfanilamide when active Co A serves as the acetyl donor.

§ Fisher "Adsorption alumina, 80-200 mm"

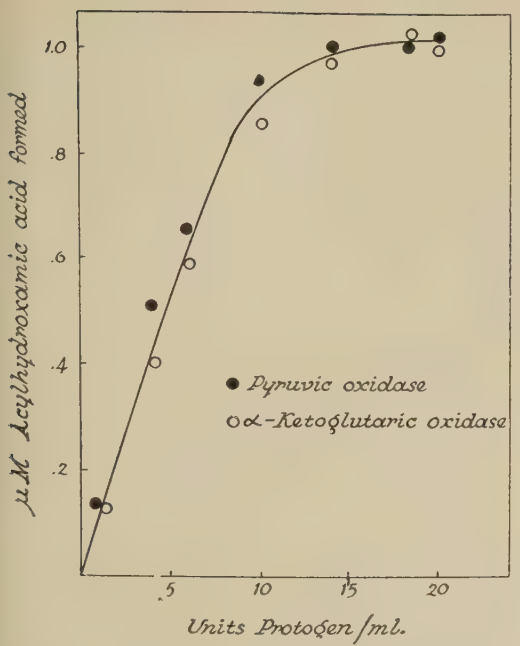

FIG. 1. Requirement of thioctic acid for formation of acyl ~ Co A from pyruvate and α-ketoglutarate. Incubation mixture was as described in the legend of Table II with the exceptions that ATP was omitted and that .4 ml of pyruvic oxidase and .62 ml of α-ketoglutaric oxidase were used. The mixture was incubated 60 min. at 25°C. Enzymes were treated with alumina.

It thus appears that transferase activity(17) with the resulting formation of acetyl ~ Co A is dependent upon thioctic acid, but that acylokinase activity(17) between the acyl carrier and the acyl acceptor is independent of protogen.

In the absence of thioctic acid the main product of pyruvate oxidation is acetate (Table V). Acetaldehyde is also formed in the course of the reaction. Diacetyl or acetoin could not be detected at any time in the incubation mixture. This is in contrast to the formation of these compounds by purified mammalian pyruvic oxidase(5,18), but is in agreement with previous investigations on *Tetrahymena* enzymes(7). As the concentration of thioctic acid in the incubation mixtures is increased, the rate of acylation is increased and the rate of acetate formation is decreased (Table VI); the rate of pyruvate disappearance is also increased. In the above mentioned experiments ferricyanide replaced the indophenol dye as hydrogen acceptor. It was thus possible to add relatively large amounts of this soluble acceptor and to allow the reactions to proceed for relatively long periods of time when measuring rates of hydrogen transfer. The increased rate of hydrogen transfer in the presence of increased amounts of thioctic acid is not interpreted as being at variance with the data presented in Table I. In the presence of necessary cofactors for both components of the oxidation, an additional pathway of utilization is made available, which is reflected as the increased rate of removal of substrate and must, of course, be accompanied by a corresponding increase in hydrogen transfer.

Discussion. The finding that thioctic acid is necessary for acyl transfer to Co A is consistent with growth studies carried out with this factor. Since acyl ~ Co A serves as the carrier for such acetyl acceptor systems as citrate(19), acetylcholine(20), and other acetylated amines(21), as well as for succinyl acceptor systems(22), it is not to be expected that acetate alone would serve completely to

TABLE IV.

Enzyme treatment	Sulfanilamide removed, μM
0	.14
0 + 20 units/ml thioctic acid	.16
Alumina	.11
Alumina + 20 units/ml thioctic acid	.11

Acetyl ~ Co A as acetyl donor in acetylation of sulfanilamide. The complete system contained 100 μM phosphate (pH 7.4), 20 μM $MgCl_2$, 10 μM cysteine, .2 μM acetyl ~ Co A, .2 ml of sulfanilamide acetylase in a vol of 1 ml. Incubation was for 30 min. at 32°C.

TABLE V.

	Pyruvate disappearance, μM	Ferricyanide reduction, μM	Acetate formed, μM	Acetaldehyde formed, μM
Observed	1.36	2.54	1.24	.13
Theory		2.72	1.36	
% theory		94	91	

Pyruvate oxidation balance. Reaction mixture contained 10 μM of pyruvate, 20 μM of $MgCl_2$, 50 μg of DPN, 200 μg of cocarboxylase, 200 μM of phosphate (pH 6.8), 20 μM potassium ferricyanide, and .4 ml of enzyme in a final vol of 2 ml. Incubation time was 60 min. at 25°C. Enzyme treated with alumina.

TABLE VI.

Thioctic acid conc., units/ml	Pyruvate disappearance, μM	Ferricyanide reduction, μM	Acetate formed, μM	Sulfanilamide disappearance, μM
20	1.12	2.03	.42	.68
10	1.06	2.08	.47	.54
5	.87	1.69	.56	.30
1	.79	1.52	.72	.08
0	.74	1.26	.68	.02
Blank*	.00	.03	.04	.00

* (20 units thioctic acid but 3 mg crystalline egg albumen in place of enzyme.)

Effect of thioctic acid on pyruvate balance. Mixture contained 20 μM $MgCl_2$, 200 μg cocarboxylase, 50 μg DPN, 60 units of Co A, 200 μM phosphate (pH 7.2), 10 μM of pyruvate, 25 μM of cysteine, 3 μM of sulfanilamide, 20 μM of potassium ferricyanide, .2 ml *Tetrahymena* oxidase and .2 ml of sulfanilamide acetylase in a vol of 3 ml. Incubation time was 60 min. at 25°C. Enzymes were treated with alumina.

replace the factor in the growth of *Tetrahymena*. It would also be expected that, as shown by Dewey *et al.*(23), growth of *Tetrahymena* in protogen-deficient medium results in the accumulation of pyruvate and of α-ketoglutarate in the medium, in the same manner that thiamin deficient cultures accumulate pyruvate(24).

Since protogen is apparently essential for acylative activity, but not the hydrogen transfer mechanism, it is obvious that the formation of the primary product of pyruvate and α-ketoglutarate oxidation is independent of a protogen requirement. This is in substantial agreement with the results of Schweet, *et al.*(18) which indicate that the first product of the oxidation is an activated C_2 fragment (or in the case of α-ketoglutarate oxidation, an activated C_4 fragment). This intermediate then either undergoes oxidation to acetate or succinate, or reacts with Co A to form acyl ~ Co A. Since the intermediate is an acyl compound, it appears that protogen is concerned only with the transfer, and not the formation of acyl groups.

Summary. 1. When examined in a purified oxidase preparation from the ciliate *Tetrahymena pyriformis* S, thioctic acid is required for acyl transfer from pyruvate and α-ketoglutarate, but not for oxidative activity as measured by reduction of 2,6-dichlorophenolindophenol. 2. The end product of the oxidative phase of pyruvate metabolism is acetate. However, a small amount of acetaldehyde is formed from pyruvate via a side reaction. It is probable that the product of α-ketoglutarate oxidation is succinate and that succinic semialdehyde is formed as a side reaction of this oxidation. 3. Thioctic acid is not required for the acetylation of sulfanilamide from acetyl~Co A.

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Effect of Some Steroids and of Corticotropin (ACTH) on Cellular Activity. (20062)

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The writer pointed out that adrenal cortical extract suppresses the increased capillary permeability induced in inflammatory states by leukotaxine or by the unfractionated crude inflammatory exudate(1). The same type of inhibitory effect was found with Compound E or cortisone(2). Subsequently it was found that the suppressing capacity of cortisone is only effective against an alkaline exudate or leukotaxine, whereas in the case of an acid exudate the hormone was ineffective. Corticotropin (ACTH), was found capable of repressing another factor present in acid exudates also capable of increasing capillary permeability(3,4). In this way two factors were shown to exist in inflammatory exudates capable of increasing permeability, namely an initial substance, leukotaxine, and another factor termed exudin(3-5). Leukotaxine has the additional property of inducing leukocytic migration, but exudin merely causes increased capillary permeability in the later stages of an acute inflammation without essentially displaying any chemotactic effect towards leukocytes. Subsequent studies have indicated that cortisone and ACTH are capable of localizing from the circulating blood into an area of inflammation(6-8).

It is of importance to determine or at least attempt to throw some light on the mechanism of suppression by cortisone and ACTH in inflammation. The present report indicates that cortisone and corticotropin (ACTH), as well as some other hormones derived from the adrenal cortex, suppress cell activity. On the contrary, another steroid which is concerned in protein anabolic processes, testosterone, and a growth-promoting hormone of the pituitary, somatrofin (STH), have no such effect in inhibiting cellular activity. Cellular activity has been measured on Arbacia ova by their ability to segment following fertilization. If the ovum is adversely affected by a given steroid, the incidence of cleavage or developmental capacity is correspondingly reduced.

Method. The first series of experiments on sea urchin ova were performed by the addition of 2.5 mg of commercial cortisone acetate (Merck) suspended in about 0.1 cc of its vehicle in a Syracuse dish containing 10 cc of sea water. A batch of ova in approximately 0.5 cc of sea water was then added. An interval ranging between 15 to 20 minutes or so elapsed before the addition of 5 or 6 drops of sperm suspension. The interval prior to the addition of the sperms thus gave the ova sufficient time to come in contact with the cortisone suspension. For a period of one hour and a half to about 3 hours later, 50 ova taken at random were counted. The eggs which showed some degree of cleavage were recorded, and the eggs which failed to display any segmentation were also noted. In another Syracuse dish containing 10 cc of sea water but no cortisone, the same procedure was followed to control for the results obtained with cortisone. As a *control* for the vehicle in which cortisone is suspended, a few experiments were performed with the filtrate obtained when the cortisone suspension was filtered through ordinary filter paper (Whatman No. 5). Several experiments were also set up in which the ova were exposed to 0.1 cc of the commercial vehicle utilized in the prep-

aration of commercial cortisone. The vehicle was originally obtained through the kindness of the Merck Co. It contains the following ingredients:

.9% benzyl alcohol
.9% sodium chloride
.4% polyoxyethylene sorbitan monooleate
.5% sodium carboxymethyl cellulose (distilled water, q.s. ad 100%)

A *third series* of experiments served to determine the effect of the residue of cortisone acetate following filtration of the suspension on the incidence of cleavage of Arbacia ova. The residue was taken up in approximately 0.2 cc of sea water and added to 10 cc of sea water in a Syracuse dish. The subsequent procedure of adding ova and of fertilizing them was similar to the one described above. Finally, the effect on the *fertilizing capacity of sperms* was observed. Sperms were placed in contact with 2.5 mg or 5 mg of cortisone suspension in 10 cc of sea water for periods ranging from 22 to 47 minutes, and 5 or 6 drops of these cortisone-exposed sperms were then added to untreated ova. The number of ova in cleavage was subsequently counted. To be certain that the ova themselves were not damaged by any cortisone carried over with the drops of treated sperm suspension, eventually untreated normal sperms were also added, and readings on the cleavage of the ova were subsequently recorded. *Compound F* or 17-hydroxycorticosterone was suspended in 0.25 cc propylene glycol and the effect on the development of the ova was also recorded. It is the belief by many that Compound F is considerably more active than cortisone.

The question arose as to the effect of another adrenal cortical hormone, namely desoxycorticosterone. Both the glucoside and the acetate in concentrations ranging from 2.0 to 2.5 mg were assayed on the ova. The desoxycorticosterone glucoside was a Ciba preparation whereas the acetate was a Schering preparation. Upon addition of 2.5 mg of desoxycorticosterone acetate in 0.5 cc of propylene glycol to 10 cc of sea water, a white precipitate formed which was probably calcium or magnesium or perhaps both. The effect of propylene glycol was also determined in order to have a control for the vehicle utilized with desoxycorticosterone acetate and Compound F (Table II). It became of interest to determine whether the effects of the steroids of the adrenal cortex were specific or whether any other steroids would have the same effect on the cleavage of fertilized Arbacia ova. For this reason, experiments were set up to determine the effect of 2.5 mg of testosterone propionate (Ciba) on the cleavage of fertilized Arbacia ova. Has ACTH only an effect on the adrenal cortex, or has it also a direct effect? The evidence previously obtained(4,5,8) and unpublished studies on adrenalectomized rats, indicate that some ACTH (corticotropin) can apparently have a direct effect. In an endeavor to find out whether this fact could be demonstrated with isolated cells, studies on the segmentation of Arbacia ova were performed. Two preparations of ACTH were utilized: The preparation made by the Armour Co., and the preparation made by the Organon Co. The Armour ACTH was taken in sea water in doses ranging from 2 mg to 5 mg. In the case of Organon preparation, the ACTH was dissolved in the vehicle supplied along with the hormone. The solvent is water containing 2.0% glycerine, and 0.5% phenol as a preservative, adjusted to approximately pH 3.0. This vehicle alone was added to the control samples. The doses of Organon ACTH were approximately of the same magnitude as those used with the Armour preparation.

To ascertain that the effects obtained with ACTH were quite specific, another anterior pituitary hormone was assayed. This was somatrofin (STH). Concentrations of 2.5 mg STH were utilized in the experiments undertaken. The addition of this hormone to sea water induced the formation of a large precipitate. In this way it could be seen whether the effect of the solubility or insolubility of a substance was of importance in the final observations. In the foregoing experiments ACTH and somatrofin had been utilized. Therefore, to rule out the effect of proteins or protein derivatives on fertilized ova, a series of experiments were accordingly set up as follows: In 4 experiments, besides 10 cc of sea water, each contained 2.5 mg of the pseudoglobulin-albumin fraction of acid inflammatory exudates. This fraction contains crude exudin(3,4,5); in 2 experiments, 2.5 mg

TABLE I. Effect on Cleavage Development of Adding 2.5 mg of Cortisone Acetate in .1 cc of Vehicle to Suspension of Fertilized Arbacia Ova in 10 cc of Sea Water.

Exp. No.		Exposure Hr	Exposure Min.	No. of ova in cleavage	No. of ova not in cleavage
2	Exp.	1	37	0	50
	Control	1	32	49	1
4	Exp.	2	51	15	35
	Control	2	51	49	1
5	Exp.	2	25	4	46
	Control	2	35	48	2
6	Exp.	2	05	8	42
	Control	2	12	41	9
7	Exp.	1	40	14	36
	Control	1	45	49	1
8	Exp.	1	50	11	39
	Control	1	55	48	2

of the leukocytosis-promoting factor of inflammatory exudates (LPF) was used; in 4 experiments, 0.5 cc of the aqueous diffusate obtained after dialysis of either canine or rabbit inflammatory exudate was utilized; and finally in 2 experiments, 0.5 cc of the diffusate of canine blood serum was employed. These particular diffusate fractions were all Biuret positive and the diffusate fractions from inflammatory exudates were in each case also Ninhydrin positive. This indicates that these fractions were protein or at least protein derivatives.

Results. The addition of cortisone acetate to sea water, and the subsequent exposure of Arbacia ova to this hormonic preparation results in a suppression or an appreciable reduction in the number of ova capable of segmentation following their fertilization. These effects are conspicuous when the incidence of cellular division is compared to that observed in the controls. The data of 8 such experiments are assembled in Table I.

The results with cortisone are not referable to the vehicle in which the commercial cortisone acetate is suspended. The absence of any effect on the cleavage pattern can be observed with either the filtrate of the cortisone preparation utilized or with the commercial vehicle employed. The averages of the data are presented in Table II.

The cortisone residue obtained as a result of filtration, however, tends to suppress the incidence of cleavage. Such residue should not be suspended in distilled water, for in the latter medium the cortisone residue tends to sink to the bottom of the dish. Even though the distribution of the hormone is not homogeneous, sea water is found a more effective medium for the cortisone residue than is distilled water. The average data on these experiments are shown on Table II.

Are the sperms likewise affected by cortisone acetate or can the effect be only detected in the fertilized ova? When Arbacia sperms are in contact with cortisone for a variable amount of time ranging between 22 to 47 minutes at concentrations of either 2.5 mg or 5 mg of the hormone suspension per 10 cc of sea water, it is found that on transferring such sperms to a dish containing normal ova in 10 cc of sea water, the fertilizing capacity of such sperms is wholly absent. None of the ova show any subsequent segmentation. The average data and the number of experiments appear in Table II. The ova have not been affected by transferring 5 to 6 drops of sperms from the cortisone-containing dish, for if now one adds normal untreated sperm, the ova proceed to be fertilized and to segment in the usual fashion. It would seem that cortisone *per se* affects cellular activity irrespective of whether one is dealing with ova or with sperms.

Compound F or 17-hydroxycorticosterone suspended in propylene glycol likewise has the same suppressing tendency on the incidence of normal cell division in fertilized ova.* The concentration of this hormone was 5 mg in 10 cc of sea water and 0.25 cc of propylene glycol was employed in each case. In all these experiments whether with cortisone or Compound F or with other hormones of the adrenal cortex when cleavage did occur, even the cell division pattern was often pathological in nature. Abnormal or atypical cleavage with unequal division was of frequent occurrence. The average data on Compound F are summarized in Table II.

Desoxycorticosterone seems to have a similar suppressing tendency on the incidence of

* Recent unpublished studies indicate that Compound F acts on capillary permeability in inflammation just like cortisone. This hormone represses leukotaxine, but not exudin, which in turn is repressed by ACTH. Compound F was obtained through courtesy of Merck Co.

TABLE II. Composite Data on Effect of Various Fractions of Cortisone Acetate and of Other Substances on Cleavage Development of Fertilized Arbacia Ova.

Type and amt of material employed	No. of exp.	Avg No., of 50 exp. ova counted, found in cleavage	Avg No., of 50 control ova counted, found in cleavage
.20-.5 cc filtrate of cortisone acetate	3	48	49.3
.1 cc vehicle in which cortisone acetate is suspended	3	47	48
.1 cc suspension of residual fraction of cortisone acetate following filtration of 25 mg of steroid preparation	4	36	48.25
2.5-5 mg cortisone on fertilizing capacity of sperms	3	0	41.3
5 mg compound F in propylene glycol	4	23.5	46.2
.25 cc propylene glycol *per se*	2	43.5	44
2-2.5 mg desoxycorticosterone glucoside	6	27.4	46.9
2.5 mg desoxycorticosterone acetate	2	.5	46
2.5 mg testosterone propionate	8	45.4	46.4
2-5 mg ACTH (Armour preparation)	6	29.5	47.6
2.5 mg ACTH (Organon preparation)	6	21.5	41.7
2.5 mg somatrofin (STH)	4	43.6	45
2.5 mg exudin (impure)	4	41.6	43.1
2.5 mg leukocytosis-promoting factor (LPF) of canine exudate	2	38	36
.5 cc of canine or rabbit diffusate from inflammatory exudates	4	42.7	45.3
.5 cc of diffusate of canine blood serum	2	45	44

cleavage in fertilized ova. When only 2 mg of desoxycorticosterone glucoside per 10 cc of sea water was utilized this suppressing tendency seemed to be transitory; but when a slightly higher concentration of 2.5 mg was employed the suppressing effect seemed irreversible. The toxic effect was even more pronounced in the case of desoxycorticosterone acetate. One wonders whether this is wholly due to the effect of the hormonic preparation or whether the formation in the sea water of a white precipitate, which is possibly calcium, upon addition of this acetate hormone preparation, may also not have had some effect on the ultimate results. The averages of the data are accordingly summarized in Table II.

Does any steroid have a similar toxic effect on the incidence of the cleavage pattern in fertilized Arbacia ova as the hormone of the adrenal cortex? To test this point, a series of experiments were undertaken by exposing ova to 2.5 mg of testosterone propionate in 10 cc of sea water. A summary of the average data appears in Table II. It is quite clear that this steroid, which is also important in protein anabolic processes, has no effect in restraining normal segmentation in fertilized sea urchin ova.

Recent studies(7,8) have indicated that corticotropin (ACTH) penetrates from the circulating blood directly into an acutely inflamed area and once there it exerts a suppressing effect on the inflammatory reaction. As pointed out at the time(7,8), one does not deny the well known physiological mechanism in the mammalian organism of an action of this pituitary hormone on the adrenal cortex; but as employed in a pathological process, such as inflammation, where there is a marked local increased capillary permeability, some and only some ACTH from the circulation seems to seep through the capillary wall and there appears to have a direct effect on the inflammatory reaction (7,8). This viewpoint, reached as a result of numerous experiments on rabbits and dogs(7, 8), and in adrenalectomized rats, was studied again with Arbacia ova. In this set up, there is a system of isolated cells. Has ACTH any direct effect on the incidence of the cleavage pattern in a way similar to cortisone or Compound F? The procedure adopted has been

described in the section on *Method*. Generally speaking, as the average data on Table II indicate, ACTH in concentrations varying from 2 mg to 6 mg seems likewise to have a suppressing effect on the incidence of cleavage of fertilized Arbacia ova. Here also, whenever cleavage did occur, this was frequently characterized by unequal or an atypical cleavage pattern. In several instances the effect of corticotropin (ACTH) was sometimes transient and after several hours most of the ova were in some state of cellular division. In other experiments the damaging effect on the incidence of cleavage seemed irreversible. It would seem that ACTH has essentially the same direct damaging effect on the fertilized Arbacia ova as the hormones of the adrenal cortex described above. Two experiments were performed with 5 mg of Armour ACTH preparations, which also indicated that this hormone in 50% sea water likewise tends to injure Arbacia sperms, so that their capacity to fertilize normal ova with the subsequent usual cleavage pattern was appreciably diminished.

Is the effect of corticotropin (ACTH) quite specific or would another anterior pituitary hormone behave in a similar manner? The somatrofic growth hormone, somatrofin (STH), was obtained through the courtesy of Frank W. Horner, Limited, of Montreal. 2.5 mg of STH was added to 10 cc of sea water. A white suspended precipitate resulted. The fertilized ova in contact with this suspended hormone were not suppressed in their segmentation (Table II). Consequently, it is clear that a protein pituitary growth hormone has no apparent effect on the division of the ova. The effect of ACTH seems therefore, to be a direct one and it seems to be quite specific. Furthermore, the insolubility resulting in a suspended STH precipitate has no significant effect on the incidence of the normal cleavage pattern. This is important in connection with the powerful effect of the insoluble cortisone suspension on the frequency of the absence of cleavage in sea urchin ova (Tables I and II).

To rule out any protein effect or effects caused by derivatives of proteins, the ova were exposed to several fractions derived from inflammatory exudates. The data are shown in Table II. The pseudoglobulin-albumin fraction of acid exudates which contains exudin, the substance that induces increased capillary permeability in the later stages of an acute inflammation(3), was incapable of altering the usual cell division of ova. Concentrations of 2.5 mg of that fraction in 0.25% sea water placed in 10 cc of sea water failed to suppress cell division. The leukocytosis-promoting factor (LPF) which is an alpha globulin recovered from inflammatory exudates(9) likewise in 2.5 mg concentration failed to alter the cleavage pattern. Finally, the diffusate fractions of exudative material or of blood serum were ineffective in suppressing the incidence of the cleavage of ova. These diffusate fractions, as pointed out above, contained probably protein derivatives alongside with inorganic elements.

Discussion. The foregoing experiments indicate that various steroids from the adrenal cortex, such as cortisone, Compound F, and desoxycorticosterone directly affect the fertilized ova of *Arbacia punctulata* so that the incidence of normal cleavage is considerably reduced. The same direct effect is obtained with corticotropin (ACTH). With this pituitary hormone the incidence of normal cleavage is either retarded or suppressed. In many instances with either hormones of the adrenal cortex or with corticotropin (ACTH) even when cleavage occurs, the cleavage pattern tends to be abnormal in the form of unequal or partial cleavage. All these substances are considered to be protein catabolic agents. On the other hand another steroid, testosterone propionate, or the anterior pituitary growth hormone, somatrofin (STH), fails to alter the normal cleavage pattern of fertilized Arbacia ova. These latter substances are considered to be promoters of protein anabolism. The cellular division of the fertilized ova can be considered to be an index of normal cellular activity. It, therefore, would seem reasonable to assume that cortisone, and ACTH which penetrate into an acutely inflamed area from the circulation owing to an increase in local capillary permeability(6-8) exert their suppressing effect on inflammation by directly reducing cellular activity. At present investigation are underway in an endeavor to deter-

mine whether cortisone and ACTH suppress some or any of the biochemical factors formed during the course of development of an acute inflammation, and which in turn are responsible for some of the biological manifestations of inflammation(9). Studies to be reported *in extenso* elsewhere indicate that the direct injection of Compound F into an inflamed area either suppresses the formation of leukotaxine or at least tends to inactivate this substance, and also the presence of this steroid in the area of inflammation inhibits apparently the formation of the LPF (Leukocytosis promoting factor). Both of these important biological substances in inflammation are presumably formed by the injured cell(9). It would thus seem as if the mechanism of suppression by the anti-inflammatory substances is at a cellular level.

Previous studies have pointed out that an extract of the adrenal cortex would retard growth of the chick embryo(10). Karnofsky and his collaborators(11) have shown the retarding influence of cortisone and of Compound F on the growth of the chick embryo. The view that the presence of cortisone and ACTH in an inflamed area may suppress cellular activity in that area is therefore a distinct possibility, and the present studies substantiate this view.

Summary. 1. Cortisone acetate (Merck) (2.5 mg in 10 cc of sea water) tends to suppress the incidence of normal cleavage in fertilized *Arbacia punctulata* ova. This is not referable to the vehicle in which the steroid is suspended. The vehicle does not alter the cleavage pattern. Filtering the suspension of cortisone acetate yields a cortisone residue capable of suppressing the incidence of normal cleavage, whereas the filtrate which is essentially the suspending vehicle has also no such inhibitory effect. The sperms are likewise inactivated by cortisone acetate (2.5 mg or 5 mg in 10 cc of sea water). Compound F (5 mg in 10 cc of sea water) suspended in propylene glycol suppresses the incidence of normal cleavage of the fertilized ova. 2. Desoxycorticosterone acetate and the glucoside tend to have the same inhibitory effect on the cleavage of ova. 3. Two to 6 mg of ACTH (corticotropin) (Armour and Organon products) directly affects the cleavage pattern of Arbacia ova. There is also a greater incidence of suppression or at least retardation of segmentation of the ova. This indicates that this pituitary hormone can likewise have a direct toxic effect on the ova without the mediation of the adrenal cortex. 4. In contrast, a protein anabolic steroid, testosterone propionate (Ciba), in 2.5 mg concentration, has no effect on the incidence of normal cleavage in Arbacia ova. 2.5 mg of Somatrofin (STH), (a Horner product), a growth hormone from the anterior pituitary has likewise no effect on the cleavage pattern. 5. The suppressing action of ACTH is not a protein effect and it is not due to any differential solubility of the hormone. 6. The specific results are discussed from the standpoint that the suppression of cellular activity, as measured by cell division, by cortisone, Compound F, and ACTH may throw light on the mechanism of suppression at a cellular level by some of these hormones in inflammation as reported in 1940 and 1942 by the writer.

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Nutritional Factors in Hemodynamics: III. Importance of Vitamin C in Maintaining Renal VEM Mechanisms.* (20063)

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It has been observed that the vit. C deficient guinea pig has a reduced reactivity of the terminal arterioles and precapillary sphincters to topically applied epinephrine(1) and a diminished capacity to withstand hemorrhage(2). Moreover, plasma from hemorrhaged, deficient animals is devoid of vaso-excitor material (VEM) as determined by bioassay of blood plasma by the Chambers-Zweifach rat mesoappendix test(2). To obtain further understanding of the relationship of vit. C and renal VEM, we have studied the ability of kidney tissue of ascorbic acid supplemented and deficient guinea pigs to form this principle *in vitro*. It was found that during the early stages of vit. C deficiency, there was a failure of the renal VEM mechanism in contrast to the absence of any such impairment in the pair fed supplemented control animals.

Methods. Thirty albino guinea pigs, selected at random, weighing 240-560 g, were given a stock ration known to be deficient in ascorbic acid. These animals were divided into 2 groups of 15 each, one of which was given a daily oral supplement of 20 mg of ascorbic acid (20 mg of ascorbic acid in 1 cc of 0.5% sodium bicarbonate). The vit. C supplemented animals were pair fed as controls with the remaining group of 15 animals which received no supplementation. This program was followed for a total of 21 days. At intervals of 7 days (on the 7th, 14th and 21st day), 5 guinea pigs from the deficient and 5 from the treated group were sacrificed by a blow at the base of the skull. The kidneys were removed immediately, decapsulated and placed in chilled saline. Slices of cortex were obtained from both kidneys (with one exception—one pair fed control had a single usable kidney, the second being hydronephrotic), and were washed in Ringer-phosphate solution. Two hundred to 300 mg of tissue were incubated in Ringer-phosphate solution of 37.5°C under 100% oxygen for one hour.† A second tissue sample from each animal, weighing 300-400 mg was incubated in similar manner under 100% nitrogen. In each case, the Ringer-phosphate medium was decanted and freed of suspended particles by centrifuging. Bioassays for vasoactivity were made on the first Ringer-phosphate wash of the fresh tissue slices and on both the aerobic and anaerobic incubation media by means of the Chambers-Zweifach mesoappendix test(3,4).

Results. Rat tests on the nitrogen incubation media revealed a sharp contrast between the kidneys of vit. C deficient guinea pigs and those of their pair fed controls. Renal tissue from the latter group invariably produced readily demonstrable VEM activity throughout the 21-day interval. Occasional moderate weight loss resulting from necessary restriction of dietary intake had no influence on the renal elaboration of this factor. A considerable difference was encountered, however, in the guinea pigs deficient in vit. C. Of the 5 animals sacrificed at the end of 7 days, the renal tissue of 2 animals was incapable of producing VEM activity; the remaining 3 animals' kidney tissue formed demonstrable amounts of this principle. Of the 5 deficient animals sacrificed after 14 days and the 5 sacrificed after 21 days, none were found whose renal tissues released VEM. The animals maintained for 21 days of vit. C deficiency lost a moderate amount of weight in contrast to their pair fed controls; the pigs maintained for a 14-day deficiency period showed no weight loss (with one exception), yet the renal tissue

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† Each tissue sample was incubated in Ringer-phosphate solution, which was proportioned one part tissue to 5 parts Ringer-phosphate by weight.

TABLE I. Result of Rat Mesoappendix Bioassay of Kidney Slices Incubation for Vasotropic Activity in Vitamin C Deficient Guinea Pigs and Their Pair-Fed Vitamin C Supplemented Controls.

No. of animals	Days on diet	Avg original wt	Avg final wt	% wt change	σ of % wt change	O_2 kidney incubation	N kidney incubation
			Vit. C deficient				
5	7–8	365.2	375.2	+ 2.7	7.4	N	1 neutral, 1 VD, 3 VE grade 1-3+
5	14–15	366	359.6	— 1.7	11.9	N	1 VD, 4 neutral
5	21	387.6	309.2	—20.2	5.0	N	1 " 4 "
			Pair-fed vit. C supplemented controls				
5	7–8	398.4	361.4	— 1.2	7.2	N	5 VE grade 1-3+
5	14–15	415.2	414	— .3	7.4	N	5 " " 1-2+
5	21–23	400.8	427.2	+ 6.8	7.7	N	5 " " 1-3+

VE = Vasoexcitor; VD = Vasodepressor; N = Neutral.

of both groups was similarly incapable of VEM production (Table I).

Guinea pig kidney slices incubated in oxygen produced neither vaso-inhibitor nor vaso-excitor material regardless of the duration of the diet period and of the diet administered (supplemented or vit. C deficient). It was observed early in this study that the Ringer-phosphate wash of kidney slices from both the deficient and supplemented animals produced no vascular response in the rat test, irrespective of the humoral characteristics of the anaerobically incubated tissue. For this reason bioassay of the Ringer-phosphate wash of fresh kidney tissue was discontinued after 6 such tests revealed no vasotrophic activity.

Discussion. In a previously reported study, Lee and Holze have shown that vit. C deficient guinea pigs subjected to hemorrhagic shock had blood plasma in which VDM was the predominant vasotrophic material. This prevalence of VDM in the plasma was thought to have been of such concentrations as possibly to mask the presence of any significant amount of VEM. Plasma from other scorbutic animals showed a complete absence of vaso-excitor activity in the rat test ("neutral" reaction), thus extending as a second possibility the failure of any renal VEM mechanisms(2). The present studies show that early in deficiency of ascorbic acid, there develops an inability of the kidney cortex to form VEM *in vitro* during anaerobiosis. Other studies have shown that disturbances of VEM production are not limited to a single specific dietary deficiency, however; renal tissue from rats on a "cirrhotic diet" fails to elaborate this factor(5,6).

During aerobic metabolism, renal tissues release little, if any, VEM(7). With the onset of anaerobiosis, however, the renal cortical parenchyma in dogs, rats, rabbits and man begins to elaborate VEM in relatively large amounts(7,8). The release of this principle under such conditions has been compared favorably to the "Pasteur" effect, with the chief metabolic cycles utilizing primarily anaerobic enzyme systems(9). The absence of VEM formation by anaerobic renal cortex from vit. C deficient animals suggests very strongly that these enzyme systems in the kidney are greatly impaired by deficiency in ascorbic acid.

Conclusions and summary. 1. Compared with pair-fed vit. C supplemented controls, *in vitro* studies of renal tissue from guinea pigs on a vit. C free diet revealed a significant impairment of VEM forming capacity in 7 days. 2. Complete loss of *in vitro* renal VEM elaboration was found on the 14th day as well as on the 21st day of vit. C deficiency. 3. Impairment of renal VEM mechanisms was unrelated to loss of body weight. 4. The significance of these observations is discussed and related to other findings in hemorrhagic shock and vit. C deficiency in hemorrhagic shock.

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Origin of Dog Serum Cholesterol Esterase.* (20064)

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Recent reports(1-4) have presented evidence for the existence of esterifying and hydrolytic cholesterol esterase systems in pancreas, dog serum, and rat intestinal mucosa. The enzymes occurring in these tissues appeared to be identical in that they required bile salts for activity, were inactivated by heating at 65°C for 15 minutes and had the same optimum pH for the hydrolytic and esterifying systems. The identity of the enzyme in rat intestinal mucosa was further verified by the demonstration that 95% depancreatized rats showed a marked lowering of cholesterol esterase activity.

In studies by Sperry and coworkers(5,6) on the cholesterol esterase of serum, it was reported that the esterification of free cholesterol occurred when human or dog serum was incubated alone. The esterification reaction in these sera was inhibited by bile salts, but in dog serum as the bile salt concentration was increased hydrolysis of the cholesterol esters of serum took place. In a previous study(3), the serum of 5 species were examined for cholesterol esterase activity. In dog serum, but not in human, rat, rabbit or guinea pig, serum highly active cholesterol esterase systems possessing the properties of the pancreatic type enzyme could be demonstrated. The occurrence of this enzyme in the serum of the dog is unique. Since a considerable amount of controversy exists regarding the occurrence and nature of serum cholesterol esterase, it was felt that a study should be conducted to ascertain more conclusively the origin of dog serum cholesterol esterase. Depancreatized dogs were prepared and the esterifying cholesterol esterase activity was followed before and after the operation with and without the addition of raw pancreas to the diet.

Procedure. Three normal healthy mongrel dogs were used. The animals were depancreatized according to the technic of Markowitz(7). Following the operation the dogs were maintained on 10 units of insulin, ½ lb raw lean beef, 10 g sucrose and 50 g raw pancreas given twice a day. The dogs were allowed to recuperate for 2 weeks. The raw pancreas was withdrawn from the diet after this period and given again after 10 days. Blood samples were obtained before the operation and then at regular intervals. Cholesterol esterase activity was measured according to the technic previously described(2) using cholesterol-oleic acid as the substrate.

Results. The results of the experiment are shown in Table I. Prior to the operation all dogs showed the presence of an active choles-

* Reviewed by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

TABLE I. Cholesterol Esterase Activity in Serum of 3 Dogs Before and After Pancreatectomy. Enzyme digests were prepared as previously described(2). Substrate for esterification, cholesterol, 25 mg, oleic acid, 54.8 mg. pH of digests, 6.2. Bile salt solution, 1 cc 10% sodium taurocholate. Enzyme, 2 cc of dog serum.

No. of dog	Days after operation	Raw pancreas in diet	Esterification* 24 hr, %
1	Pre	—	39.1
	14	—	.9
	16	—	.7
	20	—	.0
	24	+	1.0
	28	+	1.0
2	Pre	—	17.2
	14	—	.7
	16	—	.0
	20	—	.5
	24	+	.3
	28	+	2.0
3	Pre	—	
	14	+	40.0
	16	+	1.0
	20	—	.8
	24	+	2.0
	28	+	.5

* The values obtained after the operation can be attributed to the limit of error of the method(2).

terol esterase in serum. Upon the removal of the pancreas, no activity could be detected. The feeding of raw pancreas did not produce any change, possibly because gastric acidity inactivated the enzyme(8).

Summary. Pancreas appears to be the sole source of dog serum cholesterol esterase.

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Toxoplasmosis. II. Intra-Uterine Infection in Dogs, Premature Birth and Presence of Organisms in Milk.* (20065)

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(Introduced by Albert B. Sabin.)

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Toxoplasmosis is known to be a congenital disease in the human infant. The disease has been studied in mice by Eichenwald(1), who described *in utero* infection and milk transmission. Our previous study of 2 toxoplasmosis epizootics in dogs in which pups were stillborn, born prematurely or died shortly after birth prompted this investigation. This report describes experimental congenital toxoplasmosis in dogs associated with premature birth and the presence of toxoplasma in the milk.

Materials and methods. Five healthy virgin bitches 1.5 to 2 years of age and a 3-year-old male dog were isolated for 3 weeks during which time they were treated for internal and external parasites and immunized against distemper. Diet consisted of commercially prepared cereal dog food supplemented by horse meat in a ratio of 3:1. Two preinoculation serological studies conducted prior to estrus according to the method of Sabin and Feldman(2) revealed no toxoplasma antibodies. Toxoplasma organisms of canine and human origin were used in the serological studies. The male and female were allowed to copulate daily throughout the course of the estrous cycle. Sterile caps, masks, gowns, gloves and boots were worn by all who entered the room.

* This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service.

TABLE I. Premature Birth Caused by Toxoplasma.

Dog No.	Day of gestation when inoculated	Inoculum	Toxoplasma origin	Duration of gestation,* days
227	52	2.25 ml perit. exud. I.V. 10 ml pooled brain, liver, spleen and lung emulsion subcut.	Human	58
877	51	1 ml perit. exud. I.V.	Canine	56
607	50	1.25 " " " I.P. 1.25 " " " orally	"	54
638	30	1 " " " I.V. 1 " " " I.P.	"	35
24	51	2 " " " I.V.	Porcine	56

* Normal gestation period for dogs is 61-63 days.
I.V. = intravenous; I.P. = intraperitoneal; Perit. exud. = toxoplasma infected mouse peritoneal exudate.

The room floor and cages were scrubbed daily with 5% cresol solution. The inocula consisted of peritoneal exudate and/or a pooled emulsion of brain, heart, lungs, liver, spleen, and kidneys from toxoplasma infected mice. All emulsions were prepared using 1 g of tissue in 5 ml of isotonic saline. Of the 5 bitches inoculated one received toxoplasma of human origin, 3 the parasite of canine origin, and one organisms isolated from swine(3). At parturition 2 pups and their respective placentae from each of 4 bitches were collected in sterile containers while the surviving litter mates were allowed to nurse their mothers. The mammary glands of the bitches were washed with soap and water, dried, bathed in 70% alcohol and milk samples withdrawn. Doses of 1 ml were inoculated intraperitoneally into each of 4 white Swiss mice. The pups aseptically collected at birth were necropsied and portions of the brain, thoracic and abdominal exudate, heart, lungs, liver, spleen, kidneys, and lymph nodes were obtained in a sterile manner. Each tissue was separately emulsified in isotonic saline using one part tissue to 5 parts saline. One to 3 ml of each organ emulsion was inoculated intraperitoneally into each of 3 mice. A total of at least 24 mice were used per newborn animal. The placentae were treated in a similar manner.

Results. Table I summarizes the stage of gestation when inoculated, type of inoculum, origin of toxoplasma, and duration of gestation.

Clinical signs in bitches. After an incubation period of 3 to 5 days all except one bitch manifested visible signs of disease. The bitch (No. 227) which received toxoplasma of human origin developed a sanguineous diarrhea on the third day after inoculation. This was immediately followed by a mucoid ocular discharge, serous nasal discharge, progressive sensitivity to lumbar palpation, extreme depression, anorexia and anemia. Although parturition extended over a period of 6 hours leaving the mother extremely weak, she gave birth to 6 live pups without assistance. Two of the newborn were collected aseptically and sacrificed as they emerged from the birth canal, 2 died within one hour after birth and 2 were allowed to live with their mother. After close observation for 4 days one of the 2 remaining pups became depressed, inactive, and remained separated from its mother and litter mate for long periods of time. These signs as well as distended abdomen, anorexia and periodic tenesmus were manifested until death on the sixth day. The bitch had ample milk and showed concern over her young at all times. The sixth or remaining pup showed similar signs on the seventh day and died at 9 days of age. Toxoplasma was isolated from 2 pups collected at the time of birth, from 2 pups living 6 and 9 days respectively, and from the mother's milk.

One of the 3 bitches (No. 607) inoculated with toxoplasma of canine origin was asymptomatic and gave birth to 8 apparently healthy pups. Even though the pups appeared healthy

TABLE II. Isolations of Toxoplasma by Mouse Inoculation.

Bitch No.	No. of pups in litter	No. of pups toxo isolated	Milk	Ovary of bitch	Fetal membranes and allantoic fluid
227	6	4	Pos.	S.L.	*
877	5	5	"	"	*
607	8	7	"	"	Neg.
638	6	5	*	Pos.	Pos.
24	3	2	Pos.	"	Neg.

* Not examined.
Pos. = positive; Neg. = negative; S.L. = still living.

at birth all 8 died of toxoplasmosis within 2.5 months. Two died at 12 days of age, one at 13 days, one at 14 days, one at 15 days, one at 63 days, one at 68 days, and the final pup died when 72 days of age. Since toxoplasma was isolated from 7 of 7 pups tested by mouse inoculation and no bacterial, viral or other disease could be incriminated, toxoplasmosis was considered to be the cause of death. Milk from the mother on the day of parturition contained toxoplasma.

Two bitches (Nos. 877 and 638) showed restlessness, vomiting, sanguineous diarrhea, depression, sensitivity to lumbar palpation and cough. Mastitis developed in one animal (No. 877) after she gave birth to 5 pups. Two of the pups were stillborn, 2 were collected aseptically and sacrificed at birth, and one died when 4 days of age. Toxoplasma was isolated from all 5 pups and from the milk of the mother. The second bitch (No. 638) aborted 3 pups after 35 days gestation, developed convulsions and died. At autopsy 3 unborn pups were found in her uterus. Toxoplasma was isolated from 2 aborted pups, 3 unborn pups, fetal membranes, allantoic fluid and from the ovary of the bitch.

The fifth bitch (No. 24) studied was inoculated with toxoplasma of porcine origin. Four days later she developed extreme respiratory distress and pulmonary edema followed by premature birth of 3 pups and death on the fifth day. Toxoplasma was isolated from 2 of the premature pups examined. Milk collected at the time of parturition, as well as her ovaries and mammary glands collected at autopsy yielded toxoplasma.

A summary in Table II shows that congenital infection occurred in 82% of the pups born of 5 mothers inoculated parenterally with toxoplasma. Eichenwald(1) found infection in 52% of young mice born and nursed by mothers which were infected by ingestion of toxoplasma-infected mouse liver.

Upon isolation of the organism from these infected animals its identity was proven by morphologic similarity to the toxoplasma inoculated, and its pathogenicity for mice. In addition the reisolated organism was proven to be antigenically identical to known toxoplasma when employed as the antigen in the Sabin-Feldman test. It also was capable of producing specific toxoplasma antibody.

Summary. 1. Toxoplasma of human, canine, and porcine origin was pathogenic for pregnant bitches and caused abortion or premature parturition. The organism was isolated from 23 of 28 pups born of 5 bitches. 2. *In utero* transmission of toxoplasmosis was demonstrated by isolation of toxoplasma from each of 8 pups (born of 4 bitches) which were collected aseptically and sacrificed at the time they emerged from the birth canal. Isolation of organisms from the placenta and allantoic fluid was further evidence of *in utero* transmission. 3. A mammary gland emulsion from one bitch and samples of milk collected aseptically from 4 lactating infected bitches each revealed toxoplasma.

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Effect of Prior Immunization on Bactericidal Action of Penicillin *in vivo*. (20066)

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The present experiments attempt to assess the degree to which the bactericidal action of penicillin may be modified by the presence of antibodies. It had previously been shown in an early group A streptococcal infection of non-immune mice that cure was achieved only if the antibiotic effected virtual sterilization (1-3). Even when more than 99.99% of the organisms had been killed under the impact of the drug, the few organisms still surviving after the penicillin had fallen to ineffective levels regularly recovered and remultiplied to cause a fatal infection. As will be here shown, prior immunization with the homologous organism had no effect either on the rate at which penicillin killed the bacteria *in vivo*, or on the duration of its action. The outcome of treatment was, however, strikingly modified. In these partially immune mice, a small fraction of the normally curative dose of penicillin sufficed to reduce the bacterial population below the number which could be handled by the host defense mechanisms. Under these circumstances, the bacteria surviving the direct action of penicillin did not remultiply, but slowly disappeared, and the animals recovered.

Experimental. Exp. 1. One hundred white mice weighing 18 to 22 g were a) inoculated intraperitoneally with 4×10^6 group A streptococci (C-203 strain of *Streptococcus pyogenes*), and 2 hours later were treated intramuscularly in the left hind leg with 200 mg/kg of aqueous sodium penicillin G. This treatment was repeated 7 hours after inoculation. The virulence of the strain had been maintained by passage through mice twice weekly for a period of 3 years. b) The same sequence of inoculation and treatment was repeated in all the animals after 12 days. c) Eight days after the second immunizing inoculation the animals were inoculated into the right leg muscle with 4×10^6 bacteria; and in 50 of the mice, penicillin at a dosage of 12.5 mg/kg was injected 2 hours later. Groups of 6-8 mice were sacrificed at varying intervals after treatment in order to determine the number of organisms surviving in the inoculated leg muscle, as previously described(1-3).

1) *The degree of immunization* afforded by the 2 immunizing injections is shown in Table I. In this experiment, the LD_{50} was on the order of 4×10^6 organisms in the immunized animals, and approximately 10 in normal controls simultaneously inoculated.

2) *The rate of bacterial multiplication in normal and immunized mice.* When the immunized mice were inoculated with 4×10^6 organisms, the bacteria initially grew out at essentially the same rate as in normal animals (top half of Table II). After 8 hours, however, the average number of viable organisms was significantly lower in the immunized animals; and the difference between the 2 groups became progressively more pronounced. The eventual mortality in the immunized mice receiving this inoculum was 8/20.

3) *The effect of treatment* with penicillin

TABLE I. Effect of Prior Immunization with Group A Streptococci on LD_{50} Inoculum (Exp. 1).

Mice		Mortality of mice receiving indicated number of organisms intramusc.			Approx. LD_{50}
Normal	Inoculum	10^1	10^2	10^3	10
	Mortality	10/20	19/20	18/20	
Immunized	Inoculum	4×10^5	4×10^6	4×10^7	4×10^6
	Mortality	1/15	8/20	15/20	

* U. S. Public Health Service, Federal Security Agency.

TABLE II. Rate at Which Group A Streptococcus Multiply in Normal and Previously Immunized Mice.*

Exp.	Mice	Millions of organisms in inoculated muscle at indicated time (hr) after inoculation												Mortality after 28 days, untreat. mice
		0†	2		4		8		24		48		72	
1	Normal	3	25	19			313	157	346	154	154	99	576	10
		2.8	23	18			310	157	198	77	128	58	115	
		2.7	21	15			224	131	160	58	121	35	42	(3 mice
		2.7	19	11			166	112	160	15	99	22	30	dead)
		2.6												
	Immunized	2.5	24	12			64	7.1	64	7.5	27	6.2	12.8	.0037
		2	23	9.5			51	6.4	35	3	26	.032	4.9	0
		1.9	20	9.4			51	6.2	27	2.3	7.5	.024	.028	0
			15	6			43	2.8	18	.0016	6.9	.024	.0073	0
2	Normal		62	41	278	77	404	222						
		6.5	51	23	272	32	403	179						
		5.1	46		195		323							
		3.8	46		157		304							
		3.2												
	Immunized	3	72	51	200	99	426	102						
		3	62	23	179	84	336	102						
			56	20	170		266	102						
			51	13	141		144							

Exp.	Mice	Mortality after 28 days, untreat. mice
1	Normal	20/20
	Immunized	8/20
2	Normal	20/20
	Immunized	20/20

* Mice were inoculated intramuscularly with 4×10^6 organisms (by microscopic count). In Exp. 1, the plate count on the inoculum was 2.3×10^6, and in Exp. 2, 4.5×10^6 colonies.

† Immediately after inoculation there was no significant difference in the number of viable bacteria in the normal and immunized mice, and at this time period the two groups have been combined in the table.

TABLE III. Effect of Treatment with Penicillin on an Intramuscular Focus of Group A Streptococci in Normal and Partially Immune Mice.

Exp.	Penicillin dosage, mg/kg			Thousands of bacteria in inoculated muscle at indicated time after treatment (hr)								Mortality in penicillin treated mice
				0 (×1000)	1½	3	6	9	12	24	48	
1	12.5	Normal*	Range of observations†	16–51	62–92	4.2–148	11–42	124–844	1200–13100			20/20
			Mean‡	27.8	74	16.2	20	374	3460			
			S.E.(×/÷)§	1.17	1.05	1.54	1.18	1.31	1.34			
		Immunized*	Range†	20–32	8.6–34	2.9–8.8	.3–78	4 mice=0 4 mice=.05–4.7	7 mice=0 1 mouse=.05	6 mice=0 2 mice= 5.5–1000	0	0/20
			Mean‡	25.3	13.5	5.35	1.29	—	—	—	—	
			S.E.(×/÷)§	1.06	1.02	1.15	2.64					
2	3.2	Normal	Range†	7.7–23	14–52	5.2–524	6.7–322	142–7700	1280–29000	720–400000		20/20
			Mean‡	12.8	23.6	35	29.4	1120	7760	494000		
			S.E.(×/÷)§	1.17	1.24	2.1	1.86	1.91	1.72	2.76		
		Immunized	Range†		.65–118	5.2–32	.3–28	.08–32	.1–24000	2 mice=0 6 mice= 5.4–1360	2 mice died 1 mouse=0 5 mice= .05–19600	13/20
			Mean‡	12.8	19.9	12.1	1.95	2.22	4.96			
			S.E.(×/÷)§	1.17	1.9	1.25	1.74	2.1	4.53			

* In Exp. 1, normal and immunized mice were inoculated and treated on different days. In Exp. 2, the same bacterial suspension was used in both groups, and the same zero time values apply to both (*cf.* footnote †, Table II).

† 6-8 mice in each experimental group. ‡ Geometric mean.

§ S.E. (stand. error) is the antilog of the stand. error of the mean logarithm. The stand. error of the mean logarithm was estimated as range of log counts / No. of animals, after Mantel(4).

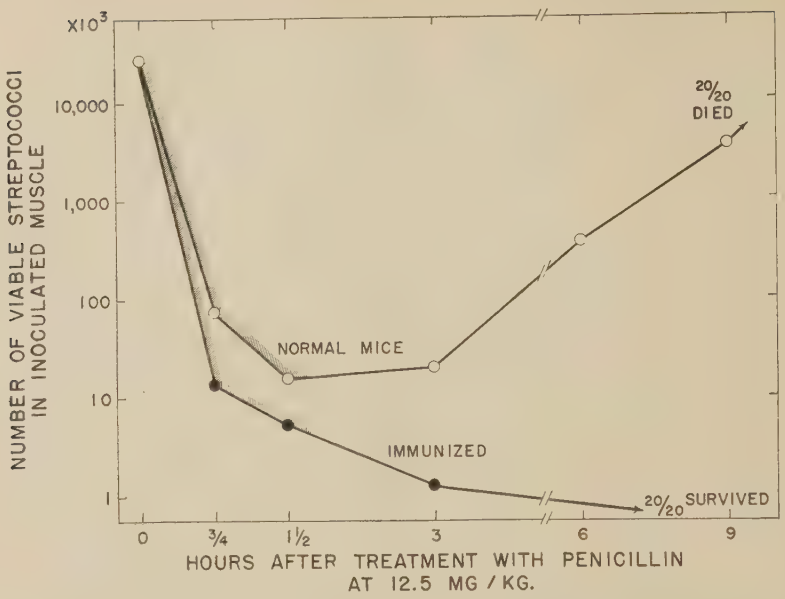

FIG. 1. The bactericidal action of a single dose of sodium penicillin G (12.5 mg/kg) in normal and immunized mice infected with group A streptococci (from data of Table III). The cross-hatched portion of the curve indicates the avg time for which the serum concentration of penicillin remains in excess of .05 μg/ml after this dosage(3).

in the immunized mice and in normal controls is shown in Table III and Fig. 1. The rapid bactericidal action of penicillin proceeded at the same rate and for essentially the same period in both groups. The single intramuscular dosage of 12.5 mg/kg of aqueous sodium penicillin used in this experiment provides a serum concentration in excess of 0.05 μg/kg for approximately 1.7 hours and in excess of 0.02 μg/kg for approximately 2.0 hours(3). These concentrations have been estimated to provide the maximally and minimally effective concentrations at the focus of infection(3); and the bactericidal effect continued for approximately the time that the serum concentration remained in excess of these levels. However, once the penicillin had fallen to ineffective levels, the course of infection deviated markedly in the 2 groups of mice. In the normal controls, all the animals treated at this dosage level died. The number of viable bacteria surviving this subcurative dose of 12.5 mg/kg remained essentially constant in number for approximately 4-6 hours, and then regularly resumed remultiplication to cause a fatal infection. In the immunized mice, however, the surviving bacteria had been reduced to a mean level of 13,500, much less than the number of organisms necessary to induce a fatal infection (Table I). As indicated in Fig. 1 and Table III, the organisms surviving the action of penicillin gradually disappeared over the following 24-48 hours, and all of 20 treated animals survived.

Exp. 2. Qualitatively similar results were obtained in a second experiment. In this experiment, 20 days elapsed between the 2 immunizing injections; and 12 days elapsed between the second immunizing injection and the final test of penicillin action. Either because of this change, or because of a difference in the condition of the immunizing culture, the immune response was much less marked in this second experiment. An inoculum of 4×10^4 bacteria killed every one of 20 animals tested, as compared with an LD_{50} of 4×10^6 in the first experiment; smaller doses were unfortunately not tried. Despite the less pronounced immunity, the results obtained were qualitatively the same as in the first experiment. For the first 8 hours, bacteria inoculated into these immunized mice grew out at exactly the same rate as in normal animals simultaneously inoculated (Table II). When

the infected animals were treated with penicillin (this time at a dosage of only 3.2 mg/kg), the bactericidal effect proceeded at the same rate and for the same period of time in the normal and in the immunized mice (bottom half of Table III). This dose of 3.2 mg/kg provides levels of 0.05 and 0.02 mg/kg for 1.1 and 1.4 hours, respectively; and the bactericidal effect of penicillin continued for approximately this period. At this point, 1½ hours after treatment, the number of organisms had been reduced to a mean level of 20,000-25,000 in both groups of animals. Once again, in all the normal controls, bacteria which survived the action of penicillin eventually recovered, and rapidly grew out to cause a regularly fatal infection. In the immunized animals, however, 7 of 20 mice were cured by the single dose of 3.2 mg/kg. (This was approximately 1/35th the dose normally necessary to cure half the animals.) The bacteria surviving the action of penicillin were gradually disposed of by the host defense mechanisms, the muscle became sterile, and the animals survived (Table III). In 13/20 of these animals, however, because of the less pronounced immunity effected in this experiment, some of the surviving organisms recovered and remultiplied to cause a fatal infection. The varying course of the infection in the animals which died and those which survived is evident in the bottom half of Table III.

Summary and discussion. In the group A streptococcal infection of mice here studied, and under the conditions of the present experiments, the presence of a significant degree of immunity had no effect on the direct bactericidal action of penicillin. This proceeded at the same rate in the immunized animals as in control mice simultaneously inoculated, and continued for the same period of time. The difference in the 2 groups lay in the course of the infection after penicillin had fallen to ineffective levels. In non-immune mice, the surviving bacteria regularly grew out to cause a fatal infection. In the partially immunized animals, however, a small fraction of the normally curative dose of penicillin sufficed to reduce the bacterial population to levels which could be handled by the host defenses. Those surviving bacteria gradually disappeared in the course of 24-48 hours, after penicillin itself was no longer operative, and the animals survived. Under the conditions of this experiment, the host defenses and the bactericidal action of penicillin proceeded independently of each other, but were mutually supplementary in effecting cure in the partially immunized animals.

The choice of a group A streptococcal infection was perhaps unfortunate. The resistance to reinfection in the immunized animals probably reflects enhanced phagocytosis; and in the present experiments, there was no pronounced cellular infiltration at the focus of infection at the time of treatment with penicillin. It would be of interest to carry out similar studies under conditions closer to those usually operative in the natural infection of man, with the cellular host defenses already mobilized at the focus of infection. It would be of interest also to determine, in infections caused by organisms susceptible to the bactericidal action of antibodies and complement, whether the presence of such antibodies modifies the therapeutic action of penicillin and other antibiotics.

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Free Amino Acids of Cytoplasmic Fractions of Liver.* (20067)

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Information on the concentrations of the free amino acids in mitochondrial preparations and other cytoplasmic fractions of liver is of interest in connection with the problem of the biosynthesis and dissimilation of proteins as well as of other problems of cellular metabolism. Such information is lacking in print. In the present investigation the concentrations of 13 amino acids have been determined by microbiological assay in mitochondrial fractions prepared in two ways and on the supernatant fluid containing microsomes from the centrifuged mitochondria. The effect of a 2-hour incubation period on the free amino acid concentrations also was determined.

Experimental. Preparation of cytoplasmic liver fractions. The livers of fed, stock rats were employed. All steps of the isolation procedures were performed in the cold at 0-5°C. Centrifugation was done in an International refrigerated centrifuge for low speeds and in a Spinco preparative ultracentrifuge for high speeds. The incubations were carried out in a constant temperature bath at 37-38°C in rotating tubes. *Buffer mitochondrial preparation (M_B).* This was prepared according to the procedure of Peterson and Greenberg (1) employing a buffer solution composed of 0.12 M KCl-0.04 M $KHCO_3$ saturated with 95% O_2-5% CO_2. *Sucrose mitochondrial preparation (M_S).* The isolation procedure was a modification of the 0.25 M sucrose method of Schneider and Hogeboom(2) designed to cut down the preparation time because of the lability of the mitochondria in amino acid incorporation experiments. The essence of the procedure was one centrifugation at 2500 r.p.m. (1100 x g) for 3 minutes to remove the nuclear fraction, two centrifugations of the supernatant fluid at 1600 r.p.m. (450 x g) for 3 minutes each, and two centrifugations of the mitochondria sediment suspended in the 0.25 M sucrose in the Spinco ultracentrifuge at 10,000 r.p.m. (6,590 x g) for 20 minutes each. The sediment, after the final centrifugation, was diluted with an equal volume of the KCl-$KHCO_3$ buffer and aerated with the 95% O_2-5% CO_2 gas mixture in preparation for incubation. The ratio of ribose nucleic acid to nitrogen of mitochondria prepared in this manner became constant

TABLE I. Free Amino Acid Contents of Cytoplasmic Fractions.

	Supernatant		Buffer-mitochondria		Sucrose-mitochondria	
	B.I.*	A.I.*	B.I.	A.I.	B.I.	A.I.
Nitrogen (γ/ml)	2.3 ± 0	2.4 ± .11	2.1 ± .04	2.2 ± .13	1.4 ± .04	1.4 ± .08
Amino acids (γ/ml)						
Alanine	6.5	11.3	1.4	5.5	1.6	4.6
Arginine	1.2	.7	1.4	2.1	.4	.7
Aspartic acid	4.1	1.9	4.2	8.8	1.3	4.8
Glutamic acid	38.9	58.2	13.6	25.9	2.6	1.9
Glycine	10.0	12.1	5.0	13.3	4.7	10.1
Isoleucine	2.0	2.0	2.2	10.8	—	—
Leucine	2.4	2.5	2.8	11.4	6.4	8.7
Lysine	3.0	4.6	2.1	8.4	1.3	2.3
Phenylalanine	1.0	1.1	1.2	6.8	2.4	4.1
Proline	1.5	2.3	1.7	3.0	3.1	2.6
Tryptophan	.3	.1	.0	.2	.2	.6
Tyrosine	1.1	.4	.7	3.8	2.2	4.1
Valine	2.0	2.3	2.5	10.3	3.3	6.3

* B.I., before incubation; A.I., after incubation.

* Aided by a research grant from the Hobson Fund for Cancer Research of the University of California.

after the first resuspension. *Supernatant (S).* The supernatant fluid from a 1:1 liver homogenate in buffer, centrifuged for 30 minutes at 4,000 r.p.m. (2700 x g) was employed for this fraction. *Preparation of filtrates for microbiological assay.* These were prepared according to Schurr *et al.*(3) by adding 2.5 ml of distilled water per ml of protein preparation in each tube and heating at 100°C for 5 minutes. The heated solutions were homogenized, and freshly prepared 10% sodium tungstate and 0.6 N H_2SO_4 (5:7) was added in the amount of 1.5 ml per ml of original protein preparation. To remove all precipitated material, the mixtures were centrifuged twice at 2000 r.p.m. (700 x g) for 10 minutes, once at 10000 r.p.m. (6590 x g) for 20 minutes and then filtered. Aliquots of the filtrates were employed for microbiological assay. The final dilution of the mitochondria and supernatant fractions was 10 times the original volumes in fresh tissue.

Microbiological assays. All preparations were adjusted to pH 7.0 and the free amino acids determined microbiologically according to the method of Henderson, Brickson, and Snell(4). A Cannon dispenser and titrator was employed to dispense the samples and media and to titrate the lactic acid formed. The samples were dispensed in volumes from 0.05 to 0.25 ml, the total volume per assay tube being made up to 0.50 ml.

The following modifications in procedure were made for assay of certain individual amino acids: For determination of alanine the samples were irradiated and B_6 emitted from the basal medium. Glutamic acid was determined after glutamine had been decomposed by autoclaving samples at 18 pound steam pressure for 20 minutes. A basal medium described by Dunn and coworkers(5) was purchased† for the determination of proline and aspartic acids. Good agreement was found for the different assay levels over the range of the standard curves of the amino acids tested.

Discussion. The data for the free amino acid concentrations expressed as γ/mg protein N, are given in Table I. The change in amino acid concentrations (determined as γ per ml, of each preparation suspended in an equal volume of buffer) during incubation are plotted in Fig. 1. The deviations from the means are represented by the vertical lines. Besides the 13 amino acids shown in the figure, determinations were also made of methionine, but

† H. M. Chemical Co., Santa Monica, Calif.

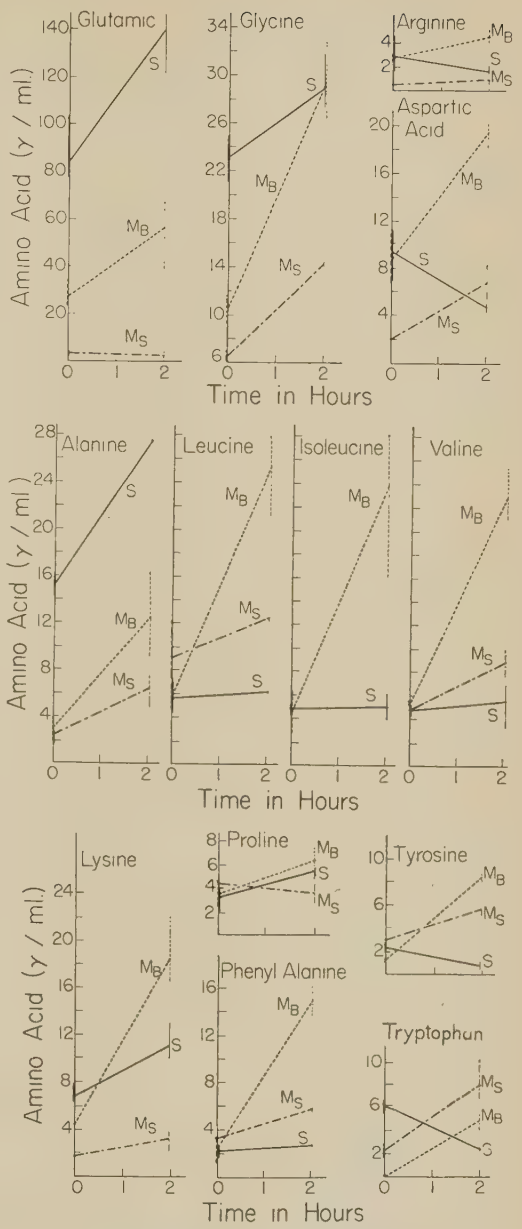

FIG. 1. Free amino acids of cytoplasmic fractions of liver before and after incubation at 37°C. S, supernatant fluid; M_B, buffer-prepared mitochondria; M_s, 0.25 M sucrose-prepared mitochondria.

the values were too low (<1 γ/ml) to be reliable and therefore are omitted. Analysis of sucrose-prepared mitochondria for isoleucine was not complete due to a variation in the properties of the organism which developed at that point.

It is clear that the concentrations of amino acids should be lower than the true values *in situ* because of the losses due to the washing procedures employed in the isolation. The sucrose preparations have a one third less nitrogen content and, in general, show a lower concentration of the free amino acids, although there is no 3:2 proportionality, as would be expected from the nitrogen analysis. Table I shows that for certain of the amino acids the concentration is the same prior to incubation for the buffer or sucrose-prepared mitochondria. In other instances the buffer-prepared mitochondria and the supernatant fluid have closely agreeing concentrations. The free amino acid concentrations in the mitochondrial preparations were calculated to range between 25 to 75% of that determined in our laboratory for whole liver homogenate.

Those amino acids which are relatively stable metabolically and are also essential amino acids (Fig. 1), or are derived from essential amino acids, increase in both mitochondrial fractions upon incubation, but remain approximately unchanged in the microsome-containing supernatant. This may indicate an extensive hydrolysis of protein within the mitochondria and but little in the supernatant and microsomal fractions. In the group of essential amino acids, a significantly greater increase occurs in the buffer-prepared than in the sucrose-prepared mitochondria with the exception of phenylalanine.

The data show that there are appreciable concentrations in the mitochondria of the 6 essential amino acids determined, as well as of the non-essential amino acids and that, with two apparent exceptions, the concentrations of the free amino acids increase upon incubation. Therefore, all the required amino acids appear to be available as substrates for the biosynthesis of proteins.

Summary. The concentrations of 13 free amino acids were determined in mitochondrial preparations of liver and in the supernatant fluid. Appreciable concentrations of the amino acids were found and, in general, these increased during a 2-hour period of incubation.

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Effects of Total-Body X-Irradiation on the Tissue Mast Cell. (20068)

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Hemorrhage is a very striking characteristic of the syndrome which follows total-body irradiation. Strong indications that hyperheparinemia is the primary cause of post-irradiation bleeding have been found in the X-irradiated dog where 1) clotting time is greatly prolonged, 2) toluidine blue or protamine administration effects normal clotting time and controls hemorrhage, 3) material having the properties of heparin appears in the blood, and 4) toluidine blue or protamine is effective in controlling hemorrhage in the presence of marked thrombocytopenia(1,2). The importance of hyperheparinemia has been questioned, however, by workers who have failed to establish prolonged clotting time as an essential component of the radiation syndrome(3-8), and the recent finding that

TABLE I.

Exp. No.	Species	No. of animals	X-ray dosage, r	Sacrifice time (days post-irrad.)
1	Hamster	16	1200	1, 2, 3, 4
2	"	37	775	3, 5, 7, 8, 9
3	"	43	600	3, 5, 7, 10, 12, 14, 17, 19, 20, 24, 26, 29, 31, 33, 35
4	Mouse	9	850	2, 5, 7

thrombocyte transfusions control hemorrhage in irradiated dogs has been interpreted as formidable evidence that thrombocytopenia is the fundamental cause of post-irradiation bleeding(9).

Reasoning that changes in circulating heparin should be reflected in the tissue mast cells, generally considered as a source of heparin(10,11), we have undertaken to determine the behavior of the mast cells in the X-irradiated animal. The present paper is concerned with the results of these studies and their possible relationship to post-irradiation hemorrhage.

Methods. The cheek pouch of the young adult male Syrian hamster and the mesentery of the hamster and of the young adult female CF#1 mouse were selected for study, since they contain mast cells in large numbers and can be observed *in toto* rather than as tissue sections. The several experiments performed are outlined in Table I. On each sacrifice day cheek pouches and/or mesenteries were removed from 3 to 5 animals under Nembutal® anesthesia. Whole mounts were prepared by placing the tissues between concentric rings (stainless steel). These were fixed for 24 hours in absolute alcohol and then treated with 0.1% toluidine blue 0 in 50% alcohol (3 hours) or with 50% alcohol saturated with thionin (10 minutes), both of which react metachromatically with heparin. On each sacrifice day tissues were also prepared from 2 or 3 nonirradiated but otherwise similarly treated controls. The *radiation factors* were 250 kv, 15 ma, 0.5-mm Cu and 3.0-mm Bakelite filters, 26.7 cm target distance, 1.5 mm Cu half-value layer, and 215-225 r per minute.

Results. The tissue mast cells of the irradiated animals presented the same appearance as those of the nonirradiated controls until 3 to 5 days after exposure when numerous atypical mast cells became apparent in the tissues of all irradiated animals regardless of the radiation dosage employed. Occurring singly or in combination, the mast cell changes consisted of 1) conglomerations of cytoplasmic granules of varying size, 2) colorless or metachromatic cytoplasmic vacuoles of various sizes, some being so large as greatly to distend the cell, 3) abnormally shaped cells, 4) discontinuities in the cell boundaries, the cell contents remaining intact, and 5) ruptured cells, the granules and granule conglomerates being scattered throughout the surrounding tissue (Fig. 1-6). The number of atypical cells increased with time, becoming greatest at 7 to 10 days post-irradiation. At this time metachromatic remnants of mast cells were present throughout the tissues, and few normal mast cells were encountered. By the 12th to 14th day after exposure the number of atypical mast cells and the amount of metachromatic material dispersed in the tissues started to decrease and continued to diminish thereafter. Simultaneously the numbers of normal tissue mast cells increased until by the 30th to 35th day, the tissues from the irradiated animals were similar to those of the controls. This picture of recovery was seen only in the 600 r group, for the higher radiation dosages were lethal, and animals were available for sacrifice only to the limits noted in experimental outline. Atypical mast cells similar to those described above were found in nonirradiated animals, but only occasionally. Attempts to determine the influence of irradiation on the number of mast cells met with little success, for distribution of the cells was not uniform, many cells being found in some areas and few in others. Observations at low magnifications gave the impression that the total number of mast cells was decreased

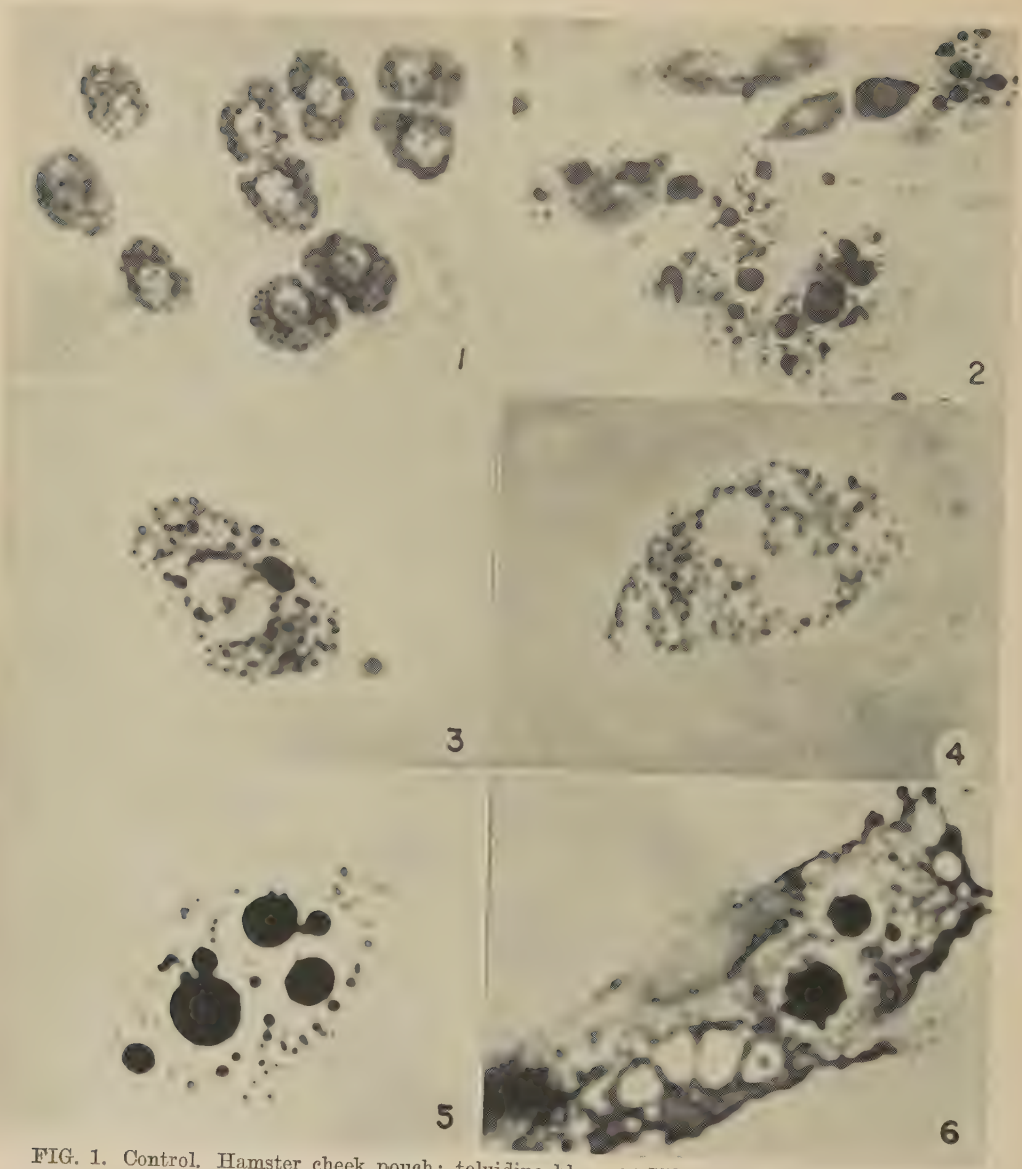

FIG. 1. Control. Hamster cheek pouch; toluidine blue; × 750.
FIG. 2. Irradiated animal. Hamster cheek pouch; 7 days post 775 r; toluidine blue; × 750.
FIG. 3-6. Irradiated animal. Hamster cheek pouch; 7 days post 775 r, toluidine blue; × 1800. Clumping of metachromatic granules, vacuolation, cell deformation, cell disruption.

when large numbers of atypical cells were apparent, but unchanged at all other times.

Discussion. The present study clearly demonstrates that total-body X-irradiation is followed by striking changes in the morphology of the tissue mast cells, the end point of which is the complete disruption of the cells with dispersion of their contents into the surrounding tissue. The probable sequence of events is 1) clumping of granules and vacuole formation, 2) increase in size of granule clumps and vacuoles, and 3) distension and rupture of the cell, allowing the granule clumps and vacuoles to be dispersed into the nearby tissues. Similar changes have been found in the tissue mast cells after a variety of treatments: allowing tissue cultures of dog mast cell tumors to age(12); administration

of cortisone(13,14), and toluidine blue(15); and stimulation of urticaria pigmentosa lesions(16). Since the several types of atypical mast cell were occasionally seen in control tissues, the results of these treatments have been interpreted as demonstrations of secretion by the mast cell. The same interpretation can be given to the present results for the same reason.

Fulton *et al.*(17) have observed that the whole blood clotting time of hamsters exposed to 1200 r gradually increases from a pre-irradiation control level of approximately 2 minutes to a value of about 5 minutes on the sixth day after irradiation. The correlation between these results and the time of occurrence of widespread mast cell changes indicates that upon release from the cell the heparin-containing material is more available to the circulation and, in part at least, can be responsible for the post-irradiation clotting defect. Previous reports on the influence of irradiation upon the tissue mast cells have been primarily concerned with changes in the cell population. The results conflict. Thus, increases(18) or lack of change(19,20), or decreases(20) in the mast cells of the irradiated skin of the rat have been noted. Increased numbers of mast cells have been reported for practically every human tissue (21,22) and for the thymus glands of hamsters(23) exposed to total-body irradiation. It is possible that species and organ differences may account for the discrepancies between these results and those of the present study. The conclusions reached in the thymus gland experiments, however, are open to question. In these studies cortisone, adrenal cortical extract, and starvation, all of which bring about thymic involution, were employed to determine whether the mast cell count would increase as the organ diminished in size. Finding no change in the cell counts under these conditions, it was concluded that the increased number of mast cells seen after irradiation was real. This conclusion seems valid until one takes note of the fact that cortisone *per se* causes depletion and disruption of the mast cells(13,14).

In the previous reports(18-23) no mention was made of atypical cells of the kind observed in the present experiments. Failure to find such abnormalities may be accounted for by the fact that the previous work was done on tissue sections, which in our experience rarely afford opportunity for visualizing the alterations readily apparent in whole mounts. The morphological changes noted in the present work have since been confirmed in the cheek pouch of the irradiated hamster by Maynard and Fulton(24).

Summary. 1. The effect of total-body X-irradiation upon the state of the tissue mast cells in the cheek pouch and/or mesentery in the hamster and the mouse was studied. 2. Following exposure there occurred in the tissue mast cells a series of striking changes the end result of which appeared to be disruption of the cell with dispersion of its contents into the surrounding tissues. 3. The possible relationships of these findings to the hemorrhage and clotting defect of the radiation syndrome were discussed.

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Ultra-Short-Acting Thiobarbituric Acids. (20069)

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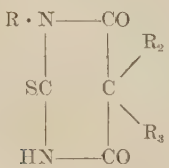
N-alkyl barbiturates were first prepared in 1904(1). The brief action of evipal, or N-methyl-cyclohexenylmethyl barbituric acid, was demonstrated in 1932(2). Subsequent workers(3,4) synthesized newer N-alkyl and N-aryl substituted barbituric acids. One of us(5) studied the series prepared by Shonle and Doran(4) and stressed the short duration of their action. American investigators(6,7) further succeeded in preparing short-acting thio-analogs of barbituric acid. Their work led to the introduction of thiopental(8,9) and other sulfur-containing barbiturates(10-13) to anesthesiology. Later N-substituted thiobarbiturates were synthesized and found to have a short induction and short duration of anesthesia. Similar work was undertaken by the Organic Chemical Division of our laboratories.



	R	R_2	R_3
[I]	CH_3	C_2H_5	$CH(CH_3) \cdot CH_2 \cdot CH_2CH_3$
[II]	CH_3	C_2H_5	$CH(C_2H_5) \cdot CH_2 \cdot CH_3$
[III]	CH_3	$CH_2 \cdot CH : CH_2$	$CH(CH_3) \cdot CH_2 \cdot CH_2 \cdot CH_3$
[IV]	CH_3	$CH_2 \cdot CH : CH_2$	$CH(C_2H_5) \cdot CH_2 \cdot CH_3$
[V]	H	$CH_2 \cdot CH : CH_2$	$CH(CH_3) \cdot CH_2 \cdot CH_2 \cdot CH_3$
[VI]	H	C_2H_5	$CH(CH_3) \cdot CH_2 \cdot CH_2 \cdot CH_3$

Forty-five new N-substituted thiobarbituric acids were made available to us for pharmacological evaluation. It was hoped that both replacement of sulfur for oxygen and alkylation of nitrogen might result in products having an even shorter action than thiopental.

Preliminary studies showed that the introduction of an ethyl or allyl radical to nitrogen gives rise to many compounds that hemolyze the mammalian blood and, following injection, cause phlebitis. A few members have convulsant action. The best hypnotic and anesthetic compounds by intravenous injection are N-methyl thiobarbiturates. It was very difficult to predict the desired activity of a product on the basis of its structure. However, four closely allied derivatives, [I], [II], [III], and [IV], appeared more outstanding than the remaining 41. More extensive experiments were therefore undertaken in order to demonstrate with greater certainty the relative activity of these compounds. In all experiments comparisons were made with sodium 5-allyl-5-(1-methylbutyl)-thiobarbiturate [V] and thiopental [VI]. The formulas of the 6 acids are as follows:

To ascertain the median anesthetic dose (AD_{50}) and median lethal dose (LD_{50}) of each compound, the solution was injected intravenously into rats, rabbits, cats, and dogs. The figures were read off from the logarithmic-probit graph paper as devised by Miller and Tainter(14). The total number of animals used was as follows: 510 rats with an

TABLE I. Comparison of AD_{50} and LD_{50} in mg/kg by Intravenous Injection.

Compound	Rats		Rabbits		Cats		Dogs	
	AD_{50} ± S.E.	LD_{50} ± S.E.	AD_{50} ± S.E.	LD_{50} ± S.E.	AD_{50} ± S.E.	LD_{50} ± S.E.	AD_{50} ± S.F.	LD_{50} ± S.E.
I	23 ± 1.8	47.5 ± 4.5	13 ± 1.2	26 ± .5	9.8 ± .45	27.5 ± 1.6	19 ± 2	38 ± 1.5
II	24 ± 1.6	51 ± 2.5	13 ± 1.2	24 ± 1.3	9 ± .54	30 ± 1.3	18 ± 1.2	43 ± 1.8
III	28.8 ± 2.5	52 ± 2.4	15.5 ± 1.2	24 ± 2.3	9.8 ± .45	31.5 ± 2	20.5 ± 1.2	39 ± 4.1
IV	23.8 ± 2.5	47.5 ± 4.5	16.2 ± 2.3	28 ± 4	10.5 ± 1	28.8 ± .7	19.5 ± 1.8	39.1 ± 1.6
V	33 ± 1.6	66 ± 2	18.1 ± 1.2	26 ± 1.4	10.4 ± .46	27.5 ± 1.6	13.3 ± .83	36.3 ± 1.4
VI	29 ± 1.5	64 ± 3	23 ± 1.6	31 ± 2.21	10.4 ± .92	32.5 ± 1.5	16 ± .97	36 ± 1.3

TABLE III. Comparison of Cumulation of Action by Hourly Injections Intravenously.

Compound	Dose, mg/kg	No. of dogs	Mean duration of action in min.					
			0 hr	1 hr	2 hr	3 hr	4 hr	5 hr	6 hr
I	10	7	10.2 ± .8	11.3 ± 2.7	17.7 ± 2.7	22.5 ± 2.7	32.5 ± 3.9	36.5 ± 4.9	43.7 ± 6.3
II	10	8	12.8 ± 1.3	14.8 ± 2.4	22.3 ± 2.3	25.5 ± 2.4	31.2 ± 3.8	36.8 ± 3.8	47 ± 5.9
III	10	5	7.8 ± 1.9	10.2 ± .3	13.5 ± 1.9	17.8 ± 1.4	28.8 ± 3	35.4 ± 6.4	52.3 ± 13.3
IV	10	4	10 ± 1.4	11.6 ± 1	16.9 ± 2	20.5 ± .5	23.7 ± .7	25.2 ± .5	27.2 ± 1
V	6.5	12	5.2 ± .7	7.2 ± 1.6	10.2 ± 2.2	24.2 ± 5.5	43.9 ± 6		
VI	8	11	4.6 ± 1.8	9.7 ± 1.8	23.2 ± 4.8	66.3 ± 11.8			

average body weight of 112 g; 160 rabbits with an average body weight of 3.04 kg; 158 cats with an average body weight of 2.31 kg; and 143 dogs with an average body weight of 5.9 kg. All compounds were in form of sodium salts. Fresh solutions, 2 or 5%, were prepared on the day of the experiment with the addition of 60 mg of anhydrous sodium carbonate to every g of the substance. Rats were used in groups of 8-10 for each dose of the barbiturates, whereas the 3 larger species of animals were employed in groups of no less than 5 for each dose.

Results. In Table I it will be noted that rats and rabbits are apparently more sensitive to the 4 N-methyl barbiturates than to the 2 non-methylated thiobarbiturates, as indicated by their LD_{50}'s and AD_{50}'s. In cats the AD_{50}'s and LD_{50}'s of all 6 compounds were approximately the same. Dogs, on the other hand, were less susceptible to the 4 N-methyl derivatives than to [V] and [VI].

In order to determine the duration of action, an AD_{60} was selected and injected intravenously. This dose anesthetized 3 out of 5 animals, and is therefore called an observed AD_{60}. Our observation extended from the completion of injection to the moment when the animal was on its feet after a period of anesthesia and sleep. The data in Table II show that the 4 N-methyl thiobarbiturates were uniformly shorter acting than compounds [V] and [VI] in all 4 species of animals. In this respect, it seems that the N-methyl thiobarbiturates substantiated our postulate that the N-methylation would have a shorter duration of anesthetic and hypnotic action than the non-methylated compounds.

In dogs receiving the AD_{50} of each product, the rectal temperature, pulse rate, and respiratory rate were recorded. The response to the 6 barbiturates was similar—namely, there was a slight fall of temperature, acceleration of heart rate, and decrease of respiratory rate. The hypothermia after the 4 N-methylated derivatives was slightly less than that after compound [V] or thiopental. In no case did the fall of rectal temperature exceed 0.78°C.

In accordance with the method of Wyngaarden and his colleagues(10), the cumulative action of the 4 N-methyl barbiturates was de-

TABLE II. Comparison of Mean Duration of Action in Minutes with an Observed AD_{60} in mg per kg after Intravenous Injection.

Compound	Rats		Rabbits		Cats		Dogs	
	AD_{60}	Duration	AD_{60}	Duration	AD_{60}	Duration	AD_{60}	Duration
I	25	32	15	26	10	26	20	38
II	25	28	15	30	10	29	20	31
III	30	23	17.5	27	10	32	22.5	29
IV	25	27	17.5	33	12.5	27	20	32
V	36.5	162	20	48	11	50	15	113
VI	30	186	25	47	11	58	17.5	142

termined and compared with that of compound [V] and thiopental. In our experiments approximately one-half of the AD_{50} was injected intravenously into groups of 4-12 dogs. The time that elapsed between hypnosis and recovery was recorded. The injection was repeated every hour until the duration of action exceeded 60 minutes. The results are tabulated in Table III. Although the duration of anesthesia with the observed AD_{60} of the N-methylated compounds was shorter than that of the 2 non-methylated products, the first injection of one-half of the AD_{50} of the latter was followed by a shorter duration of hypnotic action. The reason for this anomaly was not explored. After 7 hourly injections of the N-methyl substituted barbiturates, all the dogs showed an average duration of action less than 60 minutes. In contrast, compound [V] caused a duration of action greater than 60 minutes after the sixth injection, and thiopental after the fourth injection. If the time after the first injection for each compound is taken as unity, and if the subsequent figures are expressed in per cent of the initial time, we may plot graphs as shown in Fig. 1. Inspection of the figure makes it very clear that there is relatively less cumulation of action with the 4 N-methyl thiobarbituric acids. The mechanism of the cumulative action of thiobarbiturates has been elucidated by Brodie(15).

By the method of Burstein and Rovenstine (16) the laryngeal reflex of the cat was studied by the intravenous injection of AD_{50} of the 6 derivatives. Compounds [I] and [II] produced no effects other than central depression. Compounds [III] and [IV] induced less sneezing, coughing, and hiccuping than compound [V] and thiopental, which may be construed as manifestations of laryngeal spasm.

In 8 anesthetized dogs, 4 with ether and 4 with a barbiturate, blood pressure and respiration were recorded. The vagus nerve was exposed to electric stimulation. Small doses (2.5 to 5 mg per kg) of each barbiturate, injected intravenously, caused a fall of blood pressure with prompt recovery, accompanied by a small decrease in both the amplitude and the rate of respiration. The 4 N-methyl, as well as the 2 non-methylated, derivatives did not inhibit the vagal response.

Summary. Four N-methyl thiobarbituric acids have been studied and compared with sodium 5-allyl-5-(1-methylbutyl)thiobarbiturate and thiopental in rats, rabbits, cats, and dogs. They are all potent anesthetics by intravenous injection. The N-methylated derivatives, in an observed AD_{60}, have a shorter

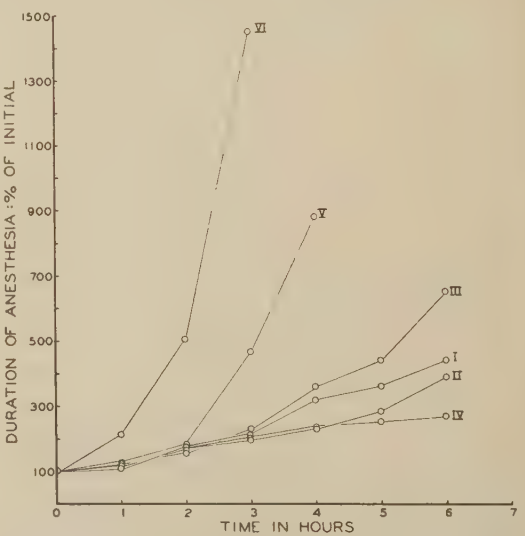


FIG. 1. Comparison of cumulative action by hourly injections.

duration of anesthetic and hypnotic action than the 2 non-methylated compounds. When one-half of AD_{50} is intravenously injected at hourly intervals, all 4 N-methyl-substituted barbiturates show less cumulative action than sodium 5-allyl-5-(1-methylbutyl)thiobarbiturate and still less than thiopental. In anesthetized cats, 2 of the N-methyl barbiturates produce less hiccup, sneezing, and coughing than sodium 5-allyl-5-(1-methylbutyl)thiobarbiturate and thiopental, while the 2 others are free from such effects. Like all barbiturates, the 4 N-methylated compounds, when injected intravenously in anesthetized dogs, lower the blood pressure and depress respiration. They do not inhibit the vagal response in these preparations. They induce slight hypothermia and tachycardia in dogs following an AD_{50} of each product.

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Escherichia coli Hemagglutinin Response of Adult Volunteers to Ingested *E. coli* 055 B_5. (20070)

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The concept of the possible etiological role of certain serogroups (0111, 055 and 026) of *Escherichia coli* in epidemic diarrhea of the newborn until recently was based largely on epidemiological data, namely, the presence in the feces of a single serogroup in a high percentage of infants with the disease in contrast to its rare occurrence in healthy infants and children suffering from other maladies. Furthermore, it is noteworthy that the particular antigenic serogroup of *E. coli* is frequently the predominant coliform organism in the feces of affected patients. Recently, additional support was gained from feeding studies, indicating that the administration of *E. coli* 0111 and 055 to adult volunteers resulted in the development of mild gastrointestinal upset to severe gastroenteritis in the majority of volunteers; in contrast, ingestion of a "normal" strain of *E. coli* was not followed by ill effects (1-3). Furthermore, ingestion of *E. coli* 0111 by an infant resulted in severe diarrheal disease, whereas a "normal" strain in like amounts was well tolerated(4). Similar ob-

TABLE I. *E. coli* Hemagglutinin Titers of Adult Volunteers Following Ingestion of *E. coli.*

Group and No. of volunteers	Ingested microorganism	Homologous *E. coli* hemagglutinin titer in prefeeding serum specimen	Incr. in titer of postfeeding serum specimen (fold)
I A 8	*E. coli* 055 (16000*)	—	>4
		1:100	16
		1:50	4
		1:100	16
		1:50	8
		—	>32
		—	>8
		1:50	8
B 7	*E. coli* 055 (5330*)	"	16
		1:100	4
		1:50	8
		1:100	4
		"	8
		—	>16
		1:100	8
C 8	Control (milk only)	"	0
		1:50	0
		1:200	0
		1:100	0
		—	0
		1:200	0
		1:50	0
		"	0
II A 7	*E. coli* 055 (1725*)	1:100	2
		"	4
		—	>8
		1:100	8
		1:400	2
		1:50	0
		1:100	0
B 8	*E. coli* 055 (143*)	"	0
		—	>8
		—	0
		1:50	4
		—	>8
		—	0
		—	>8
		—	>8
C 8	Control (milk only)	1:100	0
		"	0
		"	0
		1:200	0
		1:100	0
		1:200	0
		1:100	0
		1:50	0
III A 8	*E. coli* 055 (16275†)	"	0
		1:100	0
		1:400	0
		1:50	0
		"	0
		"	0
		"	0
		—	0
B 8	"Normal" *E. coli* #5421 (15000*)	—	0
		1:100	0
		"	0
		1:100	0
		"	0
		1:200	0
		1:100	0
		—	0

TABLE I (Cont.).

Group and No. of volunteers	Ingested microorganism	Homologous *E. coli* hemagglutinin titer in prefeeding serum specimen	Incr. in titer of postfeeding serum specimen (fold)
C 8	Control for "normal" *E. coli* #5421 (milk only)	1:100	0
		"	0
		"	0
		1:50	0
		"	0
		"	0
		"	0
		1:100	0

* Million live organisms. † Million killed organisms.
— = No hemagglutination with serum in dilution of 1:25 or higher.

servations were made on 2 additional infants(5).

The assumption of the etiological role of these serogroups of *E. coli* in epidemic diarrhea in infants would be strengthened, were it possible to demonstrate a specific antibody response during or following the illness. In the majority of infants suffering from gastroenteritis associated with these serogroups of *E. coli,* however, this has not been possible. The failure to demonstrate the development of homologous antibodies in these cases may be due to either lack of antibody response (immunogenic immaturity) or inadequate sensitivity of the conventional bacterial agglutination test. With the latter possibility in mind, a hemagglutination test was developed for the demonstration of antibodies to the serogroups of *E. coli* under discussion(6,7). It was shown that the group-specific antigens of *E. coli* 0111, 055 and 026 are adsorbed on red blood cells of various animal species; these modified erythrocytes then become specifically agglutinable by the homologous group-specific *E. coli* antisera obtained from rabbits. In order to determine whether this indirect bacterial hemagglutination test may be useful for the demonstration of *E. coli* antibodies of man, the *E. coli* hemagglutinin response of adult volunteers to ingested *E. coli* 055 B_5 was undertaken. The results of this investigation are summarized in this report.

Material and methods. All data connected with the feeding study have been described in detail elsewhere(3). It suffices therefore to briefly describe the technic used for the demonstration of the *E. coli* hemagglutinins. The pre- and post-feeding (23 days apart) sera, designated by code numbers, were examined by two of us (E.N. and N.J.Z.) and only after the hemagglutination tests had been completed, was information made available as to which sera were obtained from volunteers who had ingested *E. coli* or had served as controls. Human red blood cells of blood group O were washed 3 times in physiological saline solution. The elimination of human plasma by washing was necessary, since it was shown previously (8) that human plasma interferes with the modification of red blood cells by *E. coli* antigen. The red blood cells (2.5%) were treated with boiled suspensions of the strains of *E. coli* 055 B_5 or control strain No. 5421 which had been used in the feeding studies, according to the method described in detail previously(7). The modified cells were then washed 3 times and mixed with equal amounts (0.2 ml) of the various sera in serial dilutions. The mixtures were incubated at 37°C for 30 minutes and centrifuged at 2000 r.p.m. for one minute. The resulting hemagglutination was read grossly. All tests were carried out 3 times; the titration end points were repeatable within a 2-fold dilution range.

Results. The results of this study on the *E. coli* hemagglutinin response of adult volunteers to ingested *E. coli* are summarized in Table I. It is evident that all 15 volunteers who ingested large numbers of viable *E. coli* 055 and 10 out of 15 individuals who received smaller numbers of this microorganism responded with an increase in titer of homologous *E. coli* hemagglutinins. The specificity of this antibody response is substantiated by

the observation that there was no increase in the titers of heterologous *E. coli* 0111 and 026 hemagglutinins. Furthermore, none of the volunteers who ingested killed *E. coli* 055 organisms showed an increase in the titer of homologous antibodies, nor did the individuals who received milk without *E. coli* and served as controls. It is interesting to note also that the homologous *E. coli* hemagglutinin titers did not rise in volunteers following ingestion of living organisms of a "normal" strain of *E. coli*. Finally, it can be seen from Table I that *E. coli* hemagglutinins were present in some of the volunteers prior to the feeding of the bacterial suspensions.

Discussion. The present study was undertaken as a first attempt to determine the potential usefulness of the bacterial hemagglutination test for the demonstration of antibodies to *E. coli* serogroups 0111, 055 and 026. These serogroups are of current interest, since they are associated with epidemic diarrhea of the newborn and sporadic diarrheal disease of infants.

It is clear that the hemagglutination technic proved to be useful for the demonstration of the increase in *E. coli* 055 hemagglutinins in sera of adult volunteers who ingested live 055 organisms and that no such increase was noted in the various control groups. Obviously, any new method must be compared, if possible, with an accepted conventional technic. A comparison of the results of the *E. coli* hemagglutinin titrations, reported here, and of the conventional bacterial agglutination tests, recorded in detail elsewhere(3), leads to the following conclusions. 1) All 15 volunteers who ingested large numbers of viable *E. coli* 055 responded with an increase in the titers of *E. coli* 055 hemagglutinins; 13 of them also developed bacterial O agglutinins, 2 did not. Among the individuals who ingested smaller numbers of viable *E. coli* 055, 10 responded with a rise in *E. coli* 055 hemagglutinins and developed bacterial O agglutinins. The same 5 volunteers who did not respond with a rise in hemagglutinin titers also did not develop bacterial O agglutinins. Thus, there is a good correlation between the two tests. There is also complete agreement with respect to the results obtained in the control groups, including those who did not receive *E. coli* and those that ingested killed *E. coli* 055 organisms or living organisms of a "normal" strain. All these volunteers failed to show a rise in homologous *E. coli* hemagglutinins and bacterial O agglutinins could not be detected in the postfeeding specimens. 2) The hemagglutinin titers in the majority of postfeeding specimens of the volunteers who ingested live *E. coli* 055 organisms were approximately 5 to 20 times higher than the bacterial O agglutinin titers, indicating greater sensitivity of the former method. 3) Whereas bacterial O agglutinins could not be detected in the prefeeding specimens of any of the volunteers, the hemagglutination technic revealed the presence of *E. coli* 055 hemagglutinins in the same specimens of many of the subjects. 4) The observation that volunteers developed specific bacterial O agglutinins but not B agglutinins lends additional support to the previously made assumption(4) that the *E. coli* hemagglutination test detects O rather than B antibodies. These observations on the *E. coli* hemagglutinin response of adult volunteers to ingested live *E. coli* 055 organisms are sufficiently promising to warrant studies on infants with diarrheal disease associated with certain serogroups of *E. coli*.

Summary. The *E. coli* hemagglutinin response of adult volunteers to ingestion of *E. coli* 055 and a "normal" strain of *E. coli*, respectively, was determined. 1. Increase in *E. coli* 055 hemagglutinin titers was observed in all subjects following administration of living organisms in large numbers and in two-thirds of individuals who received smaller numbers. 2. None of the controls who received milk without *E. coli* showed such an antibody response, nor did the volunteers who ingested large numbers of killed 055 organisms or viable bacteria of a "normal" strain of *E. coli*. 3. Good correlation was found between increases of *E. coli* 055 hemagglutinin titers and the appearance of bacterial O agglutinins. 4. The *E. coli* hemagglutination test proved to be more sensitive than the conventional bacterial agglutination test, inasmuch as the former method with the postfeeding serum specimens yielded antibody titers which were from 5 to 20 times higher and

detected antibodies in prefeeding serum specimens which were not demonstrated by the latter method.

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Ultrastructure of Two Invertebrate Synapses.* (20071)

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The structure of the synapse has been the subject of numerous light microscope studies since the days of Cajal. These investigations have revealed many significant facts with regard to the grosser microscopic structural elements. However, the capriciousness with which synaptic structures appear in histological preparations has generally necessitated the use of one of the silver technics for their demonstration. These technics, while exhibiting quite well the grosser structures, appear to be subject to many severe technical limitations. They could hardly be expected to yield many significant facts about the finer structural elements intervening between pre- and postsynaptic axoplasm. Indeed, the resolution of the light microscope itself limits such studies. However, some speculation has appeared about the possible intervention of mitochondria, connective tissue, granules, or even about axoplasmic or neurofibrillar continuity(1). The high resolution obtained by the electron microscope (EM) gives promise of a more fruitful approach. Accordingly, an investigation with this instrument has been instituted. The desire to conduct correlated physiological studies and the technical limitations thereby imposed led to the choice of an invertebrate synapse for the initial work. This paper constitutes a preliminary report of these studies.

The structure of several invertebrate synapses has been the subject of light microscope investigations(2-5) and related physiological studies(6-8) have rendered plausible their division into two general types. One type is physiologically polarized, introduces an appreciable conduction delay, fatigues and responds to drugs reversibly(6). A second type is physiologically unpolarized and seems to consist of a transverse septum in certain giant fibers(6-7). Structurally both types can be shown to be variants of the same general plan of side-to-side apposition of the axons of unipolar neurones.

Materials and methods. The physiologically polarized synapse between the median giant fibers and the third motor root giant fibers of the abdominal segmental ganglia of the crayfish (*Cambarus clarkii* and *Cambarus virilus*)(6) has been the primary subject of these studies. The polarized synapse between the second order giant fiber and the third

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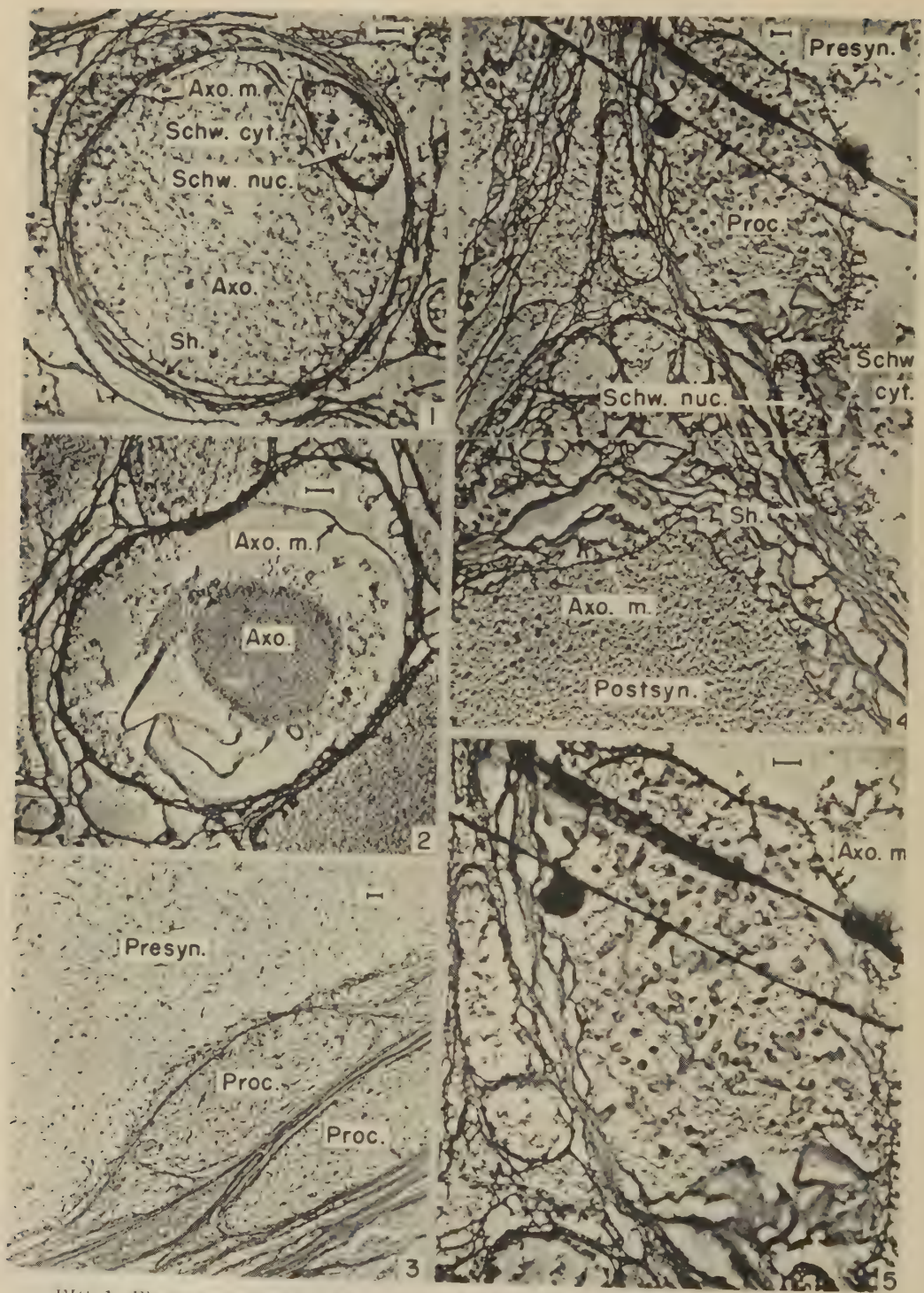

FIG. 1. Electron micrograph of a small crayfish fiber. The axoplasm (axo.), Schwann cytoplasm (Schw. cyt.), Schwann nucleus (Schw. nuc.) and the sheath (sh.) are shown. (The nucleus lying external to the sheath is of uncertain significance.) The axolemmal membrane (axo. m.) is seen separating axoplasm from Schwann cytoplasm. Osmic fixation. 4000 ×.

FIG. 2. Electron micrograph of a cross section of a small fiber in the ganglion showing the shrunken axoplasm (axo.) and the partially detached axolemmal membrane (axo. m.). Formalin-osmic fixation. 4000 ×.

FIG. 3. Electron micrograph of a cross section of the wall of the presynaptic second order giant fiber in the squid. Two synaptic processes are shown penetrating the sheath. Note the distinct difference in the concentration of filaments in the pre- and postsynaptic axoplasm. Osmic acid fixation. 2000 ×.

FIG. 4. Electron micrograph of a cross section of a portion of the crayfish synapse showing the presynaptic median giant fiber (presyn.) to the upper right, postsynaptic motor fiber (postsyn.) below, and the combined sheaths (sh.). The presynaptic Schwann cell cytoplasm (Sch. cyto.) containing a Schwann nucleus (Sch. nuc.) and a synaptic process (proc.) can be distinguished to the upper right. The axolemmal membranes (axo. m.) can be seen separating axoplasm from Schwann cell cytoplasm. Osmic acid fixation. 2700 ×.

FIG. 5. Higher magnification micrograph of the synaptic process shown in Fig. 4. 4000 ×.

order median giant fiber of the squid (*Loligo pealii*) stellate ganglion has also been studied. Fixation was accomplished by perfusion of the ganglia with 10% formalin and/or 1% osmic acid in the appropriate physiological solution (Van Harreveld's solution(9) or sea water). After thorough washing, dehydration was carried out with alcohol or dioxane according to histological methods which will be reported in detail elsewhere. The tissue was then embedded in n-butyl methacrylate. The synapse was located by the observation of serial 10-20 μ sections with the light microscope. The area of the block containing the synapse was trimmed to about 1 mm^2 and thin (circa 0.1 μ) sections were cut with the Minot International Rotary microtome(10) and a glass knife(11). The sections were mounted on 25 mesh nickel grids and the plastic was removed with toluene. After shadowing lightly with chromium the specimens were examined with an R.C.A. model EMU electron microscope.

Observations. Preliminary light microscope studies of the crayfish synapse demonstrated numerous processes of the postsynaptic fiber measuring about 0.5-5 μ in diameter extending toward the presynaptic fiber. These processes penetrate the combined sheaths of the two fibers, attain a larger diameter, up to 20 μ, and end in close apposition with the axoplasm of the presynaptic fiber in essentially the same manner as J. Z. Young had previously described for the larger (5-40 μ) processes in the squid synapse(4). In addition, a marked increase in osmiophilia of the postsynaptic axoplasm over that of the presynaptic axoplasm was noted.

The preliminary light microscope studies of the squid synapse confirmed the findings of J. Z. Young(4). The increased basophilia of the postsynaptic fibers described by Young was related to an increased osmiophilia in the crayfish.

The electron microscope studies of both the crayfish and the squid first permitted a more precise delineation of the structure of the fibers involved in the synapse. The fibers consist of a central axoplasmic core surrounded by Schwann cell cytoplasm and sheath (Fig. 1). An interfacial film between the axoplasm and Schwann cell cytoplasm, measuring about 300 Å in thickness, was observed to invest the axoplasm of the fibers. This film is considered an organized structure since at times it has been seen as a clearly defined entity stripped away from both axoplasm and Schwann cell cytoplasm (Fig. 2). It seems to be structurally related to a similar membrane first observed by Geren in myelinated vertebrate nerve(12) and to the "myelin lamellae" isolated from frozen vertebrate nerve by Sjostrand(13) and Fernandez-Moran(14). It is designated the axolemmal membrane.

The synaptic processes (Fig. 3-5) could be much more clearly differentiated with the electron microscope than with the light microscope. They are pseudopodium-like extensions of postsynaptic axoplasm and consist of densely packed axoplasmic filaments apparently completely surrounded by the axolemmal membrane of the postsynaptic fiber. They penetrate the combined sheaths of the synapsing fibers and enter the Schwann cell cytoplasm of the pre-synaptic fiber where they turn approximately at right angles and run roughly parallel to the long axis of the giant fibers (Fig. 3-6). The axolemmal membrane of the processes and the axolemmal membrane of the presynaptic fiber become closely ap-

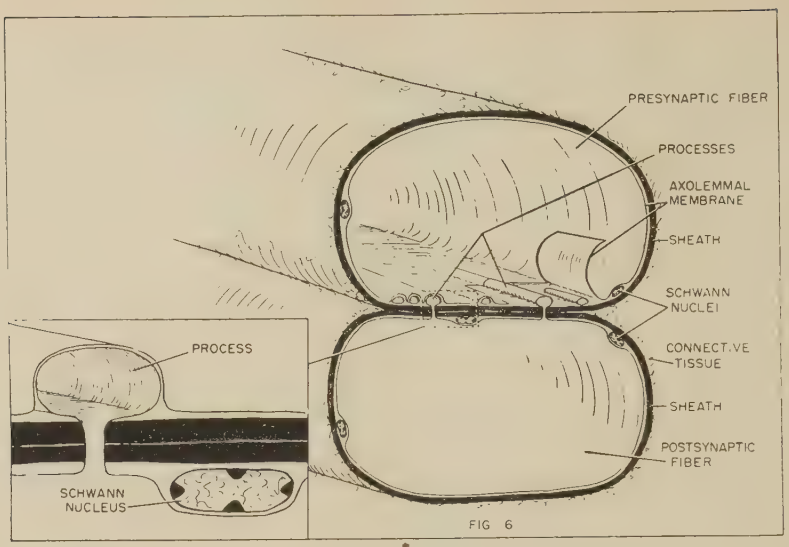

FIG. 6. A diagram of the crayfish synapse showing the presynaptic fiber above and the postsynaptic fiber below. A flap of the presynaptic axolemmal membranes is reflected to show two synaptic processes in presynaptic Schwann cell cytoplasm. The inset is an enlargement of the region designated by the dotted lines. A process of postsynaptic axoplasm surrounded by the postsynaptic axolemmal membrane is shown in presynaptic Schwann cytoplasm. The close apposition of pre- and postsynaptic axolemmal membranes is indicated.

plied to one another (Fig. 4-5). In many regions it appears that these apposed membranes fuse to form a single membrane of double thickness. However, in other regions, probably as a result of alterations associated with fixation and preparation, these closely applied membranes are separated from one another by a distance which may reach several hundred Å units. The intervening space when it was observed contained no definite structures. It is impossible to state at present with any degree of certainty whether or not such a space separates these membranes in the living synapse. But it may be stated that if such a space does exist it must be of an order of magnitude of no more than a few hundred Å units. Indeed, it is deduced from the examination of many electron micrographs that pre- and postsynaptic axoplasm are separated in the crayfish synapse by a distance of the order of magnitude of 600 Å, *i.e.*, merely by the thickness of the two apposed membranes. It is hoped that studies now in progress will lead to a more exact determination of this synaptic thickness.

The synaptic processes have consistently been observed to contain the filaments characteristic of axoplasm(15) and these filaments have provided one fairly reliable means of identifying them. Postsynaptic axoplasm, whether in the giant fiber or its synaptic processes, has been found to contain much more densely packed axoplasmic filaments than presynaptic axoplasm (Fig. 3). This increased number of axoplasmic filaments per unit volume may account for the increased osmiophilia of the postsynaptic axoplasm.

The extension of postsynaptic axoplasm toward presynaptic axoplasm and the more dense packing of the axoplasmic filaments of postsynaptic axoplasm at the synapse provide a demonstration of two types of morphological polarization which may be related in some way to the physiological polarization of these synapses.

Summary. 1. Electron microscope studies of a type of physiologically polarized synapse in the crayfish and the squid have shown that the synapse consists of a region characterized by processes of postsynaptic axoplasm lying in presynaptic Schwann cell cytoplasm. 2. The axolemmal membrane of the postsynaptic fiber completely envelops the synaptic processes and is closely applied to the correspond-

ing axolemmal membrane of the presynaptic fiber in a manner such that a single membrane of double thickness is sometimes produced. 3. Pre- and postsynaptic axolemmal membranes are structurally similar and are about 300 Å in thickness. 4. The axoplasm of the postsynaptic fiber and of the synaptic processes contains a distinctly increased number of axoplasmic filaments per unit volume over that of presynaptic axoplasm.

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Hepatic Glycogen in Acute Radiation Death.* (20072)

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In radiobiological research involving acute x-radiations, interest is directed toward discovering sensitive indicators of irradiation damage. Liver glycogen may prove to be such an indicator. It might be hoped that glycogen depletion has a quantitative relationship to irradiation dosage.

Materials and methods. Hamsters (*Cricetus auratus*), mice (Swiss albino), and salamanders (*Triturus pyrrhogaster*) were exposed to x-radiation until they were moribund. The animals were then sacrificed by decapitation, and their livers were immediately removed and fixed in cold Rossman's solution. These tissues were studied for glycogen by the histochemical technic of Hotchkiss and McManus (1,2) which involves an oxidative treatment of the sections with periodic acid and subsequent treatment with Schiff's leuco-fuchsin followed by sodium bisulfite. The specificity of the reaction is controlled by treating alternate sections with saliva or diastase before staining. As an additional check for the presence of glycogen in the hamster livers, the microchemical method of Boettiger(3) was used.

The irradiation conditions were as follows: The hamsters were killed under the 2 million volt x-ray machine made available through the courtesy of Dr. Vincent Collins of the Delafield Memorial Hospital. The dose rate was 534 r/minute at a distance of 61 cm, and the animals were moribund at 110,000 r in 3 hours and 26 minutes. The mice and salamanders were exposed between parallel tube sources at the Radiological Research Laboratory, stabilized at 110 KV and 30 MA, without filtration, with a distance of 30 cm from the source to the center of the body of the animals. The dose rate was 2200 r/minute. In all cases the animals were enclosed in plastic boxes. The salamander bodies were kept moist with cotton dampened with tap water.

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FIG. 1. Normal hamster liver, P.A.S. preparation.

FIG. 2. Liver of hamster irradiated until moribund. There is almost no Schiff positive (glycogen) material.

FIG. 3. Liver of normal salamander, P.A.S. preparation.

FIG. 4. Liver of irradiated salamander. Many hepatic cells free of glycogen. P.A.S. preparation.

Results. In the livers of those mammals irradiated as described above, there was little or no glycogen as shown by the Hotchkiss-McManus technic (Fig. 1 and 2). The microchemical determinations specific for liver glycogen confirmed this finding. The normal hamster liver contained 3% glycogen (by weight) while the liver of the irradiated animals, after only 3½ hours and 110,000 r, contained only 0.3% glycogen.

In the cold-blooded salamanders exposed to the acute lethal dose of x-radiation, there was a marked decrease in liver glycogen as illustrated by Fig. 3 and 4, but the depletion was not as complete as it was for the mammalian livers.

Discussion. The apparently contradictory report of Ross and Ely(4) that there was an increase in hepatic glycogen when fasted rats were x-irradiated up to 500 r is not applicable here since our animals were not fasted, and we used extremely high dose rate and levels of exposure. Louran and Lartigue(5) suggested that x-radiation interfered with the synthesis of glycogen in guinea pigs exposed to 500 r total body x-radiation. Their findings, however, probably have little relevance to ours, since their observations were made over a period of days and all of our animals died within a maximum of 3½ hours after the start of x-ray exposure.

The cold-blooded salamanders lived 80 minutes under a dose rate of 2200 r/minute which meant a total accumulation of 176,000 r. It is difficult to determine the death instant of these sluggish animals, and some might have "survived" further exposure under the x-ray beam. The livers of these animals did show some glycogen depletion but not as complete as that seen in the mammalian livers. This difference may be accounted for by their slower metabolic rate.

Since there was a drastic depletion of liver glycogen in all animals studied, there must be a common radiogenic cause. One cannot rule out the possibility of an adrenal-stress syndrome. There may also be an acute syndrome involving other endocrine systems such as the pituitary-pancreas-adrenal complex. A recent paper by the authors(6) points up histological manifestations of acute exposure in the lymphocytic organs, and a further paper(7) illustrates changes in the endocrine organs. It would be ill-advised to emphasize depletion of hepatic glycogen as an independent reaction. Nevertheless, the effect is so drastic and dramatic that it compares well with the known radio-sensitivity of lymphocytes.

Conclusions. In both warm-blooded animals and in cold-blooded amphibia, the liver responds to acute x-radiation by a drastic depletion in liver glycogen, as determined both by histochemical and microchemical tests.

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Effect of Chelating Agent on Urinary Lead Excretion. Comparison of Oral and Intravenous Administration. (20073)

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The need of a more effective agent for the treatment of lead poisoning is evident. The methods of therapy currently available are either of questionable effectiveness or possess serious side-effects. Recently an organic chelating agent, disodium calcium ethylene diamine tetra-acetate (CaEDTA) has been suggested for the treatment of lead poisoning and shows promise of being superior to compounds previously used(1,2). CaEDTA is stable, odorless, water-soluble, and forms a stable complex with lead which is virtually non-ionized. CaEDTA is not concentrated in any particular organs or tissues of the body, is rapidly excreted in the urine, and apparently has little or no toxicity for man in the doses used(3,4).

A study was undertaken to determine the effectiveness of the drug administered by the oral and intravenous routes. There have been no reports on the use of oral CaEDTA in humans. If oral administration were effective, it would facilitate medical control of industrial exposures to lead and would thereby broaden the usefulness of the agent.

Methods and materials. Studies on 7 patients treated with CaEDTA† are reported here (5 adults and 2 children), who had either symptoms of lead poisoning or excessive amounts of lead in their blood and/or urine by chemical determination. The *dosage* schedule of CaEDTA was arbitrary. The intravenous dosage schedule used for adults was 1.0 g on the first day and 2.0 g a day thereafter; a total of 5 treatment-days was ordinarily employed. On the first day of treatment the dose was divided into 0.2 g and 0.8 g; subsequent doses were 1.0 g given twice daily. Each dose was diluted in 250 ml of 5% glucose in water and given slowly by intravenous infusion over a period of one hour, allowing at least 6 hours between doses. Children were given a test dose of 1/10 the calculated daily dose, as were adults; subsequent doses of 30 mg/kg in 5% glucose in water (infused slowly) were given twice daily. The *oral dose* used in both adults and children was 30 mg/kg administered twice a day. For study purposes the usual course of 5 days of continuous therapy was interrupted in patients J.S., W.B., and L.Q., for one or more days to demonstrate effectiveness of the agent or to begin an alternate route of therapy. Twenty-four hour urine collections and whole blood specimens were obtained before, during, and after the treatment period for lead analysis. (The dithizone method with buffer extraction was used(5)). Multiple hemograms and urinalyses were performed on specimens from all subjects. The serum levels of calcium, magnesium, inorganic phosphorus, chloride, sodium, potassium, total protein, urea nitrogen and thymol turbidity were followed.

Results. Intravenous administration produced a 10- to 40-fold increase in urinary lead excretion on the first day of therapy. Subsequent values were generally of a lower order of magnitude but were never less than 3 times the observed pretreatment level. In contrast to the adults, maximum urinary lead excretion of the 2 children occurred on the second day of intravenous therapy, *e.g.*, the value on the second day for J.S. rose to 60 times the control value.

Oral CaEDTA produced a different pattern of lead excretion from that obtained with intravenous administration. The rise in excretion tended to be more gradual, with maximal excretion occurring on the third or fourth day of therapy. When alternate routes of admin-

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† Supplied in ampules and as tablets by Riker Laboratories, Los Angeles, Calif.

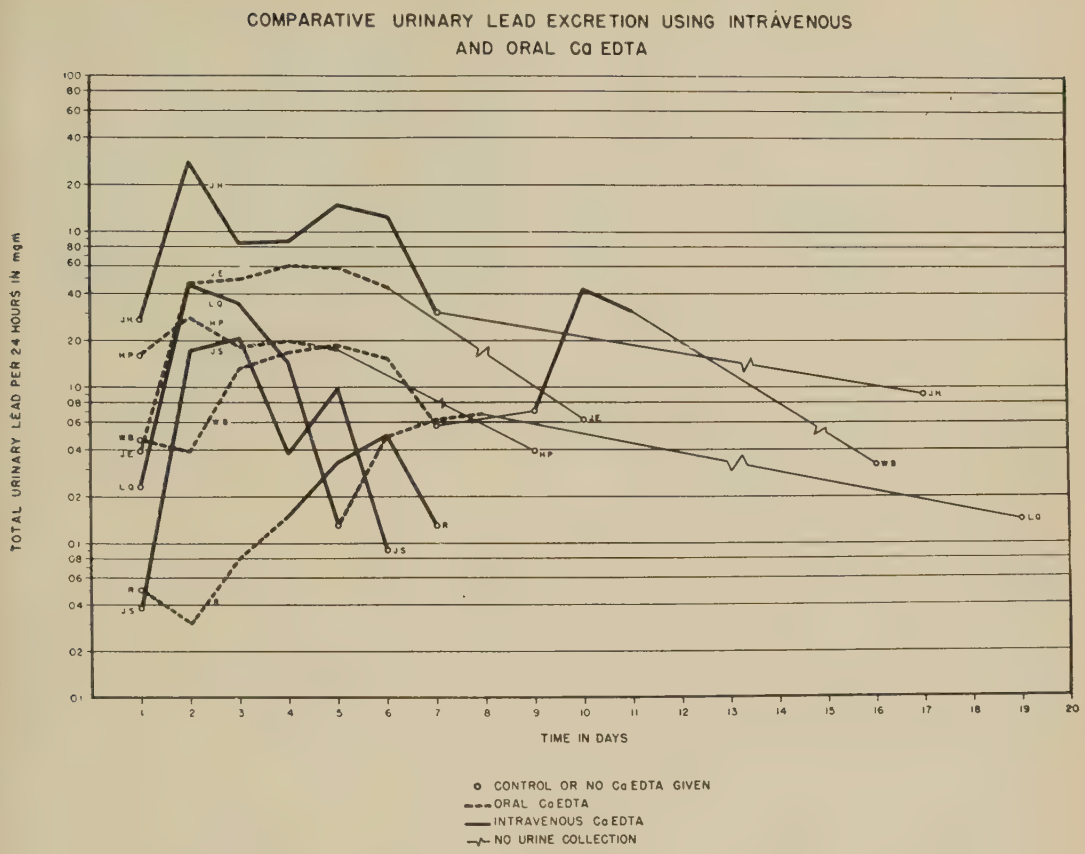

FIG. 1. The 24 hr urinary lead excretion in seven patients. A semilogarithmic scale is used for delineation of individual values. Treatment days are indicated by heavy or broken lines.

istration were used in the same individual, the maximal value of lead excretion attained with the use of intravenous infusion of CaEDTA always exceeded the maximal value obtained when oral medication was employed. Nonetheless, on the day of maximal excretion, a 5- to 10-fold rise of the 24-hour urinary lead excretion above the observed control values was attained with oral medication in all cases except J.P.

The blood lead values, stipple and reticulocyte counts showed a definite tendency to decrease. No consequential alterations were found in the chemical analyses on the serum.

There were no significant untoward effects observed when the dosage schedule given was followed. (Observations included 19 patients given CaEDTA: 4 patients followed by blood lead determinations only, 2 cases too recently treated for complete evaluation at this time; 2 "normals" given an infusion of CaEDTA, and 4 patients with other heavy metal poisonings.) Patients L.Q. and J.P. complained of malaise and light-headedness, and L.Q. also noted mild arthralgia on the first day of therapy. These symptoms, however, cleared entirely on the second day of therapy and did not recur. J.P. received 30 mg/kg of CaEDTA orally 3 times a day for the first 2 days of his 5-day course of treatment. He developed loose stools and abdominal pain after 48 hours on CaEDTA, which persisted until the medication was discontinued. This attempt to give a priming dose was undertaken when it was noted that two other patients receiving oral medication had little rise in urinary lead excretion in the first 24 hours of therapy. The desired effect was not obtained.

Discussion. The calcium chelate is used in the treatment of lead poisoning because chela-

tion by EDTA is not specific for lead. There is a definite order of chelation which varies for a given di- or tri-valent ion with the pH of the solution(3). Popovici *et al.*(6), have shown that the rapid intravenous injection of the non-chelated EDTA in rabbits produced tetany and death from hypocalcemia. By administering the compound as the calcium chelate, this danger is eliminated and toxicity is minimal.

The cases presented demonstrate that CaEDTA was well tolerated and effective in producing significant increases in the excretion of lead by the kidney. Those patients manifesting symptoms due to lead ingestion noted a prompt alleviation of symptoms. The increase in urinary lead excretion consequent to oral administration of CaEDTA was quantitatively less and maximal excretion was attained more slowly than when CaEDTA was given intravenously. Gastro-intestinal absorption of CaEDTA was suggested by the increase in urinary lead excretion following oral administration; however, the formation of the lead EDTA complex in the gastro-intestinal tract with subsequent absorption cannot be ruled out. Mosey and coworkers have shown that the lead EDTA complex when taken by mouth was rapidly absorbed from the upper gastro-intestinal tract(7). Further study is needed to clarify this point.

Summary. Seven patients with abnormally elevated blood lead and/or urinary lead excretion values are presented who received CaEDTA orally, intravenously, or by both routes. This organic chelating agent effected a marked increase in urinary lead excretion by both routes. Intravenous administration was more effective than oral therapy. Preliminary results indicate that CaEDTA is the most effective agent proposed for the treatment of plumbism. Side-reactions were minimal with the dosage schedule employed.

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Comparison of Effects of Various Phosphate Compounds and Aluminum Silicate on Isolated Frog Heart. (20074)

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Various investigators, Linder and Rigler(1), Sheikhon(2), Lichtneckert(3), Loewi(4), have noted the restorative effects of adenosine triphosphate on the hypodynamic frog heart. A great variety of surface active substances such as sodium oleate, animal charcoal, camphor and serum are also known to increase the amplitude of contraction of the hypodynamic frog heart, Wieland(5), Clark(6). The present experiments were undertaken to study the effects of various phosphate compounds, ATP, ADP and sodium tripolyphosphate(TPP) on the hypodynamic frog heart and to compare them with a surface active substance, aluminum silicate (bentonite).

Methods. The heart of a frog, *Rana pipiens,* was removed, suspended in oxygenated Ringer solution† in a glass muscle cham-

* This investigation was supported by a research grant from the National Heart Institute of the National Institutes of Health, U. S. Public Health Service.

† NaCl 0.65 g %, KCl 0.014 g%, $CaCl_2$ 0.012 g %, adjusted to pH 7.3 with phosphate buffer.

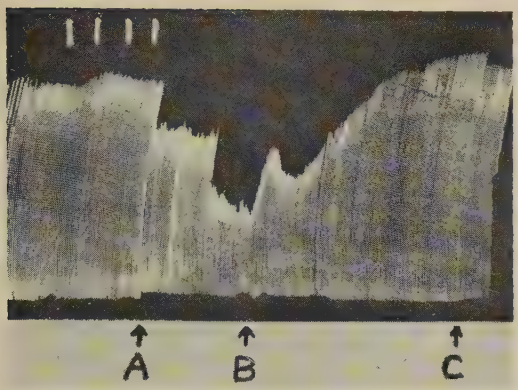

FIG. 1. Effect of ATP on rhythmically driven frog ventricle in ½ Ca-Ringer. A—addition of ½ Ca-Ringer. B—addition of .20 mM ATP. C—30 min. after addition of ATP. Time in 15 sec. intervals. Interval between A and B, 3 min. Interval between B and C, 30 min.

ber at 25°-27°, and the mechanical response of the ventricle recorded according to the method described by Clark(7). The ventricle was stimulated by shocks from a square-wave stimulator, and the contractions were recorded on a kymograph. The general procedure in all experiments consisted of 1) an initial control period of about 15 minutes with the ventricle in normal oxygenated Ringer solution, 2) a depression period in which the amplitude of contraction was lowered to 50% of the control level by one of the procedures described below, 3) a period in which the test substance was added, 4) a final period in which the ventricle was returned to normal Ringer. All values for recovery of amplitude of contraction are expressed as % of the initial control level.

Results. 24-27 experiments with each of the 4 compounds at various concentrations were done in which the ventricles stimulated at 22-60 per min were depressed by alteration of the perfusion fluid in one of the following respects: lowered calcium, increased potassium, N_2 plus IAA, or acetyl-β-methylcholine chloride. A typical kymograph record of the characteristic recovery pattern appears in Fig. 1. In general the amount and duration of recovery varied directly with the concentration of test substances added, up to a maximally effective concentration. Fig. 2 graphically summarizes the data from 24 experiments with various concentrations of ATP in the presence of ½ Ca Ringer. From curves A and B it can be seen that the degree and duration of recovery increases with increasing concentrations of ATP up to a maximum concentration of 0.19 mM. No correlation was found between the rate of stimulation and the degree of recovery. Table I summarizes the data from the 4 groups of experiments. The figures listed in this table represent average values for the degree and duration of recovery at maximal effective concentrations of the test substances.

Five experiments were done with calcium-free Ringer as the perfusion fluid. In all of these experiments the amplitude diminished to about 10% of the control level, and the addition of ATP, 0.30 mM, restored the amplitude of contraction to 80% of the control level.

The excitability of the ventricles was tested by recording their ability to respond to double the initial frequency of stimulation in the presence of Ringer solution containing six times the normal potassium content (0.084 g %). During the initial control period with normal Ringer in the cannula, the frequency of ventricular contractions followed exactly when the rate of stimulation was doubled. After replacement of the normal Ringer with the 6-fold K, the amplitude of contractions diminished and there were frequent dropped beats. With the addition of ATP (0.19 mM) or ADP (0.22 mM), the amplitude of con-

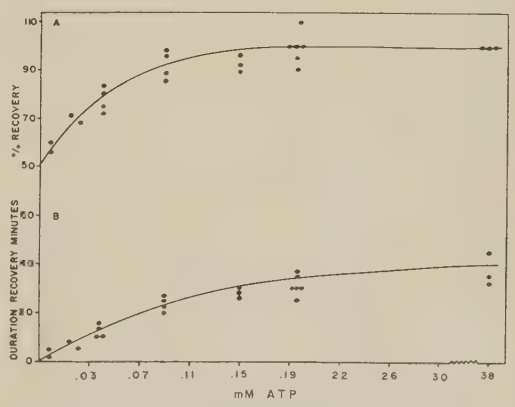

FIG. 2. Restorative effects of ATP on isolated frog ventricle in ½ Ca-Ringer. Curve A—effect of conc. of ATP on degree of recovery of amplitude of contraction. Curve B—effect of conc. of ATP on duration of recovery.

TABLE I. Comparison of Relative Effectiveness of ATP, ADP, TPP, and Bentonite at Maximal Effective Concentrations.

Solution	Test substance	Conc., mM	Recovery, %	Duration, min.
½ Ca Ringer .006 g %	ATP*	.19	100	35
	ADP*	.22	90	20
	TPP†	.27	70	90
	Bent.‡	.4 ml	85	9
4 K Ringer .056 g %	ATP	.19	100	30
	ADP	.22	79	19
	TPP	.32	80	60
	Bent.	.4 ml	60	5
N_2 + IAA 1:10000	ATP	.28	75	3
	ADP	.33	0	0
	TPP	.54	0	0
	Bent.	.6 ml	0	0
Acetyl-β-methylcholine chloride "mecholyl" 1:1000	ATP	.02	87	30
	ADP	.03	74	30
	TPP	.27	57	15
	Bent.	.4 ml	70	15

* Sodium salts of ATP and ADP were prepared by Sigma Chemical Corp., St. Louis, Mo.

† $Na_5P_3O_{10}$ kindly supplied by Victor Chemical Works, Chicago, Ill.

‡ Powdered aluminum silicate: SiO_2 43.66%, Al_2O_3 41.26%, Fe_2O_3 trace, CaO .86%, MgO .12%, loss on ignition 13.99%. Kindly supplied by Whittaker, Clark and Daniels, New York City.

traction returned to the control level and the rate of contraction followed the rate of stimulation precisely when the latter was doubled (10 exp.). TPP and bentonite were ineffective under these conditions.

All 4 compounds were effective in bringing about recovery after depression by ½ Ca, 4 K, and acetyl-β-methylcholine chloride. In general ATP was the most effective of these compounds. This observation is substantiated by the following facts:

ATP further increased the degree of recovery beyond that attained with TPP and bentonite in the ½ Ca and acetyl-β-methylcholine chloride experiments listed in Table II.

In the experiments with N_2 plus IAA, only ATP was effective in restoring contraction. Although this restoration was extremely transitory (3 minutes) it could be continued by repeated additions of ATP to the cannula. Under these conditions ATP is presumably acting metabolically by virtue of its terminal pyrophosphate. Korey(8) observed that ATP and ADP would initiate contraction of an isolated muscle fiber preparation whereas adenosinemonophosphate and inorganic pyrophosphate were ineffective. Recently, Greiner (9) reported a fall in ATP concentration concomitant with the decline of contractile force in anoxic cat papillary muscle.

ATP, and also ADP, proved effective in restoring excitability in hearts whose excitability had been depressed by excess potassium. Acierno, *et al.*(10) found that ATP restored excitability toward the normal level in hearts whose excitability was depressed by acetylcholine.

The data presented here appear to indicate that ATP can act in two ways under the conditions of these experiments, 1) by virtue of its being a linear polyphosphate and possessing a certain amount of surface activity, 2) by virtue of having a terminal "high energy" phosphate bond. Evidence for the first instance is found in the results of the ½ Ca, 4 K and acetyl-β-methylcholine chloride experiments in which ATP, ADP, TPP and bentonite all exerted restorative effects. On the other hand, in the experiments in which the energy metabolism was inhibited by nitrogen and IAA, ATP alone was effective in

TABLE II. Effects of ATP in the Presence of TPP and Bentonite.

Solution	Substance	Conc., mM	Recovery, %	Substance	Conc., mM	Recovery, %
½ Ca Ringer	TPP	.160	59	ATP	.12	88
	"	.03	52	"	.02	85
	"	.09	56	"	.06	89
Acetyl-β-methylcholine chloride "mecholyl"	"	.32	54	"	.02	78
	"	.27	54	"	.02	72
	"	.13	55	"	.02	85
	"	.03	50	"	.02	87
	Bent.	.4 ml	72	"	.24	80
	"	.2 "	59	"	.24	82
	"	.1 "	52	"	.24	82
	"	.1 "	52	"	.06	85

restoring contractions. Here, ATP was presumably acting by virtue of its possession of "energy rich" phosphate bonds.

Summary. ATP, ADP, sodium tripolyphosphate and aluminum silicate restored the amplitude of contraction in hearts made hypodynamic by reduced calcium, excess potassium, or acetylmethylcholine chloride. In general, ATP was the most effective compound. ATP alone was effective in hearts depressed by N_2 plus IAA. ATP and ADP restored the excitability in hearts depressed by excess potassium. None of the 4 compounds had any effect on the freshly excised heart perfused with oxygenated Ringer.

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Effect of Synthetic Lysine Polypeptides on Rabbit Blood Coagulation.* (20075)

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As part of studies of the biological properties of lysine polypeptides(1-5), an investigation of their effect upon blood coagulation was undertaken in 1950. Since the basic polypeptides of lysine had been shown to resemble the protamines in many of their chemical and biological properties, it was expected that they would also have an effect on blood coagulation similar to that of the protamines. During the course of our investigations, de Vries, Schwager, and Katchalski reported on their observations of the action of some water-soluble poly-α-amino acids on blood clotting (6). In view of the continued interest in synthetic polypeptides and blood coagulation (7,8), it seems desirable to report some of our results.

Experiments with synthetic polypeptides of L-lysine and L-glutamic acid were carried out to study their effects on the coagulation of rabbit blood *in vitro* and *in vivo* and compared with protamine and heparin. While human and sheep blood cells have been found to be agglutinated by very low concentrations of lysine polypeptides(3) and also protamine (9), these substances do not agglutinate rabbit red cells. Rabbit blood was therefore used because it provides a good experimental system in which to study coagulation without any complicating effects of agglutination. The results of some of these experiments indicate that lysine polypeptides prolong the whole blood clotting time, increase the prothrombin time and neutralize the effect of heparin.

Materials and methods. The polypeptides were synthesized by polymerization of the N-carboxyamino acid anhydrides and analyzed by reaction with nitrous acid to determine the average number of amino acid residues (units) per polypeptide molecule(10,11). Stock solutions were made up in 0.85% saline,

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adjusted to pH 7.0 and stored at 4°C until needed. Clotting times were determined on blood obtained from fasted anesthetized (Nembutal) adult rabbits. Blood was taken by intracardiac puncture and immediately added in 0.8 ml volumes to test tubes (9 × 75 mm) containing 0.2 ml of the test solutions. The tubes were closed with paraffined corks and inverted every half minute during the first 15 minutes, and every one or 2 minutes thereafter. The clotting time was recorded as the time when all the blood first clotted and remained in place when the tube was inverted. Control whole blood clotting times generally ranged from 4-6 minutes. However, to facilitate comparison of all the results, the clotting times were calculated so that the control in each experiment had a clotting time of 5 minutes, and the experimental clotting times were proportionally adjusted. The *prothrombin time* determination (12,13), was carried out at 37°C. Oxalated rabbit blood was centrifuged, and the plasma diluted to 12.5% by adding physiological saline and the test substances. Calcium chloride solution (0.02 M) was mixed with a thromboplastin preparation (Difco Bacto-Thromboplastin) and 0.2 ml of this mixture was added to 0.1 ml of the diluted plasma. The time from this addition to the formation of a visible fibrin clot was recorded as the prothrombin time.

Results. The effect of the 19 unit L-lysine polypeptide *in vitro* on the clotting time of rabbit blood is shown in Table I. These data show that 125 μg of the L-lysine polypeptide prevented clot formation and as little as 31 μg increased the time of clotting from 5 to 13 minutes. The lysine polypeptide was somewhat more effective than protamine. The amino acid L-lysine and the L-glutamic acid polypeptide had no effect on the clotting time.

Chargaff(14) has shown that protamine neutralizes the anticoagulant action of heparin. The data of Table II show that the lysine polypeptide also neutralizes heparin, for the clotting time was restored to normal when 62.5 μg of lysine polypeptide or protamine were added to blood containing 50 μg of heparin per ml. At higher levels, the clotting time was again prolonged. The amino acid L-lysine as well as the synthetic L-polyglutamic acid had no effect on heparin activity.

TABLE I. Effect of Lysine Polypeptide and Protamine on Blood Clotting Time.

Final conc., μg/ml	Clotting time in presence of—	
	19 unit polypeptide min.	Protamine min.
250	>40	>40
125	>40	22
62.5	18	9
31.2	13	8
0	5	5

TABLE II. Anti-Heparin Activity of Lysine Polypeptide and Protamine.

Final conc.		Clotting time in presence of	
Heparin, μg/ml	Polypeptide or protamine, μg/ml	Polypeptide, min.	Protamine, min.
50	250	>40	14
50	125	11	—
50	62.5	6	5
50	31.5	>40	22
50	15.6	>40	>40
50	0	>40	>40
0	0	5	5

TABLE III. Effect of Synthetic Polypeptide and Protamine on Prothrombin Time.

Final conc. in .1 ml 12.5% plasma, μg	Prothrombin time, sec.—	
	19 unit polypeptide	Protamine
125	100	100
31.3	27.8	100
15.6	23.9	20.2
0	18.3	12

Previous work has shown that the optical configuration and the average chain length affects the hemagglutination(3) and anti-viral activity(2,4) of the lysine polypeptides. Chain length also influences their action on blood coagulation. The 19 unit L-lysine polypeptide at a concentration of 62.5 μg per ml prolonged the clotting time to 18 minutes, while the tube containing the 158 unit L-lysine polypeptide at the same concentration showed no clot formation for over 40 minutes. The effect of a lysine polypeptide *in vitro* upon the prothrombin time is shown in the data of Table III. These data show that both the 19 unit L-lysine polypeptide and protamine increase the prothrombin time when either is added to the oxalated plasma

before the addition of the thromboplastin-calcium chloride mixture. Both substances prolonged the clotting time beyond 100 seconds when present at a concentration of 125 μg per ml. Polyglutamic acid showed no effect.

When the glutamic acid polypeptide was added to the lysine polypeptide or to protamine, a turbid flocculent precipitate was formed which indicates combination. Sufficient polylysine or protamine was added to plasma to produce a doubling of the normal prothrombin time; the addition of the synthetic polyglutamic acid restored the prothrombin time to normal.

Protamine has been used to counteract over-heparinization and it was of interest to determine whether polylysine would also counteract the action of heparin *in vivo*. Ten mg of heparin in 2 ml of solution was injected intravenously into the ear vein of an adult rabbit and 7 minutes later a blood sample removed from the other ear failed to clot within 30 minutes. Then 5 mg of a 19 unit lysine polypeptide was injected into the heparinized rabbit. When the clotting time was tested 5 and again 150 minutes later it was normal. The order of injection was reversed in another rabbit. Here, 10 mg of the 19 unit polylysine in 2 ml was injected intravenously. This amount of polylysine did not alter the clotting time. Twenty minutes after the polypeptide injection, 10 mg of heparin was injected, yet the clotting time remained normal when tested 15 minutes afterwards.

The toxicities of the polypeptides were studied in mice and guinea pigs. Death usually followed the intravenous injection of from 0.5 to 2.0 mg of lysine polypeptides of various chain lengths per adult mouse. The mice appeared to have immediate respiratory difficulties and moved in a spasmodic manner. Death generally occurred in less than 2 minutes. Autopsies revealed some vasodilation of the lungs and the spleen appeared congested. Guinea pigs generally tolerated 6 mg of the lysine polypeptides but died when injected intravenously with 10 mg.

Discussion. Our results confirm the report by de Vries, Schwager, and Katchalski(6) that the basic lysine polypeptides prolong blood coagulation, increase the prothrombin time and counteract heparin activity. In this and, as well as in their toxicities, the synthetic lysine polypeptides closely resemble the naturally occurring basic protamines. Both readily combine with a variety of proteins through the formation of many ionic bonds between the basic groups of the polypeptide or protamine and acidic groups at the surface of the protein. The basic amino groups of the lysine polypeptide also combine with the acidic sulfonic acid groups of the heparin molecule, for on mixing solutions of polylysine and heparin a turbidity immediately develops. This neutralization of the negatively charged groups of heparin by the positively charged lysine polypeptide would seem to be sufficient to account for the anti-heparin activity of the polypeptide.

The toxicities of the synthetic lysine polypeptides in mice, guinea pigs and rabbits closely resemble those of protamine(9) both in the anaphylactoid symptoms described and the approximate lethal dose ranges noted. The close similarity in the many biological effects of protamine and the lysine polypeptides suggests that high molecular weight synthetic polypeptides may be of value as models in studies which are concerned with an understanding of the basis for some of the biological and chemical properties of naturally occurring peptides and proteins.

Summary. Synthetic polypeptides were investigated for their effects on rabbit blood coagulation *in vitro* and *in vivo*. The basic L-lysine polypeptides prolonged whole blood clotting time, increased prothrombin time and neutralized the effect of heparin. A synthetic L-glutamic acid polypeptide inhibited these effects of polylysine and protamine. In mice, guinea pigs and rabbits the lysine polypeptides produced anaphylactoid symptoms which closely resembled those of protamine toxicity. A mechanism involving a combination of the basic polypeptides with acidic protein surfaces or with heparin is discussed to explain some of these results.

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Relation of Relaxin to Steroid Ovarian Hormones on Production of Mammary Spreading Factor.* (20076)

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Hamolsky and Sparrow(1) reported that daily treatment for 13 days with a combination of 0.83 μg of estradiol benzoate, 1 mg of progesterone and 25 guinea pig units of relaxin produced greater mammary gland growth in immature ovariectomized female rats than treatment with any 2 of these hormones. Since it has been well established that estrogen and progesterone in sufficient quantities produce good mammary gland growth (see review by Folley and Malpress(2)) it was concluded by Garrett and Talmage(3) that relaxin must act as a potentiator of estrogen rather than producing specific changes attributable to relaxin. A recent investigation by Trentin(4) on the effect of relaxin on mammary gland growth in the mouse has shown that there was little or no increase in the percentage of positive mammary alveolar responses in the ovariectomized mice treated with estrogen, progesterone and relaxin as compared to those treated with estrogen and progesterone only. Earlier observations by Elliott and Turner (5) showed that the injection of estradiol benzoate and progesterone into castrate albino rats in the proper proportions caused maximum elaboration or activation of a spreading factor which could be extracted from the mammary gland.

It has been suggested that the spreading factor which can be extracted from the growing mammary gland makes possible the forward extension of the mammary duct system into the fatty pad. It was thought possible that relaxin might directly or indirectly influence the elaboration or activation of the spreading factor and thus contribute to the greater mammary gland extension observed by Hamolsky and Sparrow(1).

Methods and materials. Castrate female albino rats weighing from 150 to 250 g were employed starting on the eleventh day postoperatively. The estradiol benzoate and progesterone used was dissolved in olive oil while relaxin was prepared as an aqueous extract of swine ovaries.† Daily subcutaneous injections were continued for 10 days. On the eleventh day the animals were killed, the mammary glands removed, extracted, and the amount of spreading factor determined by a method previously described(6).

* Contribution from the Department of Dairy Husbandry, Mo. Agric. Exp. Station, Journal Series No. 1343. Approved by the Director.

† The relaxin used was generously provided by Dr. R. L. Kroc, Chilcott Laboratories, Morris Plains, N. J.

TABLE I. Effects of Hormone Treatment on Amount of Spreading Factor.

Controls and treatments	No. of animals	§	Assay of spreading factors‡ Avg final bleb, mm	Area of spread, mm², mean ± S.D.
Aqueous buffer solution, pH 6	8		16 × 17	59.7 ± 13
Castrate ♀	5†		16 × 16	47.1 ± 12.6
10 day pregnant ♀ *	"		21 × 21	192.5 ± 16.5
1 μg estradiol benzoate*	"		16 × 18	72.3 ± 13.3
5 " " " *	"		20 × 21	176 ± 16.4
5 mg progesterone*	"		18 × 19	114.7 ± 14.5
1 μg E. B. + 5 mg progesterone*	"		21 × 22	209 ± 16.9
1 " + 25 GPU relaxin	"		17 × 18	86.4 ± 14.1
5 " + " " "	"		20 × 22	191.7 ± 16.5
5 mg progesterone + 25 GPU relaxin	"		18 × 19	114.7 ± 14.5
1 μg E. B. + 5 mg progesterone + 25 GPU relaxin	"		21 × 22	209 ± 16.9

* Inserted from previous exp. for comparison (5,6).
† Castrate female rats.
‡ .2 cc extract + .1 cc Evans blue dye.
§ Avg initial bleb 14 × 14 mm.

Groups of 5 rats were treated as follows: a) 1 μg estradiol benzoate and 25 guinea pig units of relaxin; b) 5 μg estradiol benzoate and 25 guinea pig units of relaxin; c) 5 mg progesterone and 25 guinea pig units of relaxin; and d) 1 μg estradiol benzoate, 5 mg progesterone and 25 guinea pig units of relaxin.

Results. The extracts from both the 1 μg and 5 μg levels of estradiol benzoate plus relaxin showed only slightly more mammary spreading factor than the same levels of estradiol benzoate alone. The addition of relaxin to the 5 mg of progesterone failed to increase the amount of spreading factor in the mammary gland extracts. Similarly the injection of 1 μg estradiol benzoate, 5 mg progesterone and relaxin produced no increase in the amount of spreading factor in comparison with a group administered estradiol benzoate and progesterone alone (Table I).

Summary. The injection of 25 guinea pig units of relaxin daily for 10 days to castrate female albino rats as an addition to estradiol benzoate and to progesterone failed to increase, significantly, the elaboration or activation of the mammary gland spreading factor in comparison with the steroid hormone action.

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Effect of Age on Local Action of Adrenocortical Hormones.* (20077)

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The direct application to rat skin of adrenocortical extract or alcoholic solutions of several

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† We wish to extend our appreciation to Drs. Eldon Nielsen and W. J. Haines of the Upjohn Co. for the extract used in these studies.

TABLE I. Duration of Local Treatment Required to Inhibit Growth of Hair in Immature and Adult Rats.

	No. of rats	Mean duration of treatment (days) — Initial inhibition	Regrowth	Prolonged inhibition*
(a) One mg/ml conc.				
Immature rats				
Extract-treated	23	38 ±10.7†	44.6±10.0‡	62.9±15.5
Ethanol-treated	14	0	0	0
Adult rats				
Extract-treated	17	16.3± 4.8	0	16.3±19.7
Ethanol-treated	14	0	0	0
(b) .25 mg/ml conc.				
Immature rats				
Extract-treated	5	57 ± 3.0	82§	69 ±19.8
Ethanol-treated	4	0	0	0
Adult rats				
Extract-treated	3	22 ±11.2	0	29 ± 7.0
Ethanol-treated	2	0	0	0

* By "prolonged inhibition" is meant a state of retarded hair growth which lasted for a minimum of 6 consecutive weeks.
† Stand. dev. ‡ Nineteen rats showed regrowth. § One rat showed regrowth.

adrenal steroids induces inhibition of hair growth(1,2) and marked connective tissue changes(3) which are limited to the area of treatment. This report demonstrates that a longer period of treatment is required to inhibit hair growth in immature than in adult rats.

Procedure. Daily percutaneous application of hog adrenocortical extract† to immature rats was begun 3 days after birth and to adult rats at approximately 70 days of age. Control animals in both groups received comparable volumes of the solvent, 25% ethanol. Because of the difference in surface area between newborn and adult rats, an attempt was made to administer approximately the same amount of hormone per unit area of skin rather than a constant quantity to a constant area in both age groups. The volume administered was the amount required to moisten the skin of the right dorsal surface of the neck within an area bounded by the right ear, center of the scapula, dorsal mid-line and lateral mid-line. The volume applied daily to newborn rats was .02 ml. This amount was increased gradually to 0.1 ml at about 40 days of age, 0.1 ml being the volume given to adult rats from the beginning of the experiment. Since two concentrations of extract were employed, *i.e.*, the equivalent of 1 mg and .25 mg of cortisone per 1 ml as determined by the liver glycogen test, the immature rats received at the beginning of the experiment 20 or 5 μg per day which was increased to the adult dosage of 100 or 25 μg at 40 days of age. In each experiment, littermate male and female newborn rats were divided into extract- and ethanol-treated groups with adult rats being run in parallel. Adult rats and the immature rats after weaning were kept in individual cages. The patterns of hair growth on the dorsum of the neck were drawn weekly. Since the normal pattern of growth is bilaterally symmetrical, inhibition was revealed by the absence of pigment (black-hooded Long-Evans rats were used) or hair on the right side at a time when these appeared on the left. The hair was clipped weekly in adults and in the young after 35 days of age. Because of the extremely dense and rapid growth of hair on young rats, they were clipped twice weekly prior to 35 days of age in order to insure adequate moistening of the skin with the applied fluids.

Results. The greater resistance of immature rats to the local growth-inhibiting action of adrenocortical extract is shown in several ways. The mean number of days of treatment required to elicit the response in adults is less than half that required for young rats, at the 1 mg/ml concentration 16 and 38 days being required, respectively (Table I). Comparable

figures at the .25 mg/ml concentration were 22 and 57 days, respectively. The lesser effectiveness in immature rats is demonstrated also by the reappearance of hair growth in the treated area in 19 of 23 rats of the former group after an average of 44.6 days of treatment. In contrast, regrowth did not occur during treatment of any adult rats. Similarly, prolonged inhibition did not begin in the immature rats until after an average of 62.9 days of treatment as compared with 16.3 days for the adults at the higher concentration of extract (Table I).

Discussion. These experiments show that age modifies tissue response to the local action of adrenocortical hormones. When growth is retarded by the presence of excessive amounts of these substances in the tissue fluids, it appears that the action of pituitary growth hormone is antagonized. Thus, growth hormone and corticotrophin exert antagonistic effects on the growth of bone in hypophysectomized rats (4). It is possible that young rats produce relatively more endogenous growth hormone than adults, or that their peripheral tissues are more responsive to it, which would explain why the inhibitory action of adrenocortical steroids is less pronounced in immature animals.

Since adrenocortical hormones may suppress growth of hair and certain aspects of the inflammatory response by a similar mechanism, one might expect that age would modify dosage requirements in the treatment of inflammatory diseases in children.

Summary. Adrenocortical extract inhibits hair growth less effectively in immature than in adult rats.

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Effects of Intravenously Administered Poly-D L-lysine in Rats. (20078)

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The recently synthesized water-soluble basic poly-α-amino acid poly-lysine(1) has been shown to have a number of actions *in vitro*. It inhibits the formation of thrombin in shed human blood(2), it inhibits fibrinolysis in a streptokinase-profibrinolysin-fibrin system(3), it is bacteriostatic(4,5), it reduces virus infectivity(6), it agglutinates chicken red cells (7) and human red cells(8) and is a hemolytic agent(9). The natural acidic polymers, heparin and poly-D-glutamic acid, and the synthetic water-soluble acidic poly-α-amino acid, poly-L-aspartic acid, have been shown to antagonize the clot delaying(2), the antifibrinolytic(3) the agglutinating(8), and the hemolytic action(9) of poly-lysine *in vitro*.

The present study reports the effects in rats of poly-D L-lysine on blood coagulation and red cells, the clinical and anatomical picture caused by toxic doses of this substance, and the antagonistic action of acid polymers.

Materials. Poly-D L-lysine hydrochloride with an average degree of polymerization-30 was prepared from D L-lysine according to Katchalski *et al.*(1). Poly-L-aspartic acid with an average degree of polymerization-150 was prepared according to Berger and Katchalski(10). Heparin, a Lederle solution containing 10 mg sodium salt of heparin per ml having anticoagulant potency of 1100 Toronto units per ml, was used as described below. Thromboplastin was prepared from human brain (11).

Methods. Solutions of poly-D L-lysine

TABLE I. Effect of Intravenous Poly-D L-Lysine on Blood Clotting Time and Prothrombin Consumption in Rats.

Poly-D L-lysine HCl, mg/100 g body wt	Clotting time, min.	Serum prothrombin, %	No. of rats
—	1.5–3.5	0	5
8	33	30	1
2	2.0–2.5	130–190	3
1.5	1.5–3.5	100–180	3
1	1.0–4.0	85–100	2
.5	3.0–3.5	50– 52	2
.2	3	45–110	2
.05	2	0– 57	2
.025 and lower	1.5–2.5	0	3

Rats receiving 8 and 2 mg and 2 rats receiving 1.5 mg polylysine hydrochloride/100 g body wt died within 10 min. after inj. Heart puncture immediately after death. Other rats sacrificed 10 min. after inj. Heart blood obtained immediately thereafter. Plasma prothrombin varied from 130–190% (expressed in % of human plasma prothrombin).

hydrochloride of various concentrations in buffered isotonic saline were injected slowly into rat tail veins in a total volume not exceeding 0.4 ml. Blood for coagulation studies was obtained by puncture of the heart exposed under ether anesthesia, avoiding admixture with tissue juice. Prothrombin of the serum, obtained one hour after clotting, was estimated by the method of Rosenfield and Tuft, using $BaSO_4$-treated oxalated human plasma as diluent(12). Rat serum prothrombin values are expressed in percentage of normal human plasma prothrombin content, measured by the same method. Blood clotting times were measured by the method of Pohle and Taylor at 37°C(13). Red cell counts were done on blood, obtained from the tail vein, in a Spencer counting chamber averaging the count of 2 chambers. Reticulocyte counts were determined from blood smears stained with brilliant cresyl blue and expressed as % of the number of red cells. Tissues were fixed in 10% formalin, sectioned at 8 μ and stained with hematoxylin eosin. Blocks were taken from the lungs, heart, spleen, liver, kidneys, lymph-nodes, muscle, skin and brain.

Results. 1. *Effect of intravenous administration of poly-D L-lysine on blood coagulation.* The results are given in Table I. The lethal dose of 8 mg per 100 g bodyweight prolonged the clotting time markedly. Lethal doses of 1.5-2 mg and sublethal doses did not prolong the clotting time, but a definite disturbance in thrombin formation, as indicated by elevated serum prothrombin values, was observed even with doses as low as 0.05 mg per 100 g bodyweight. The rather low prothrombin value of the serum of the rat given 8 mg polylysine may have been due to the inhibitory action of polylysine on the formation of thrombin in the procedure of prothrombin determination(2). It was found that the anticoagulant effect of intravenously administered polylysine in sublethal doses of 1.5 mg per 100 g bodyweight remained detectable up to 2-3 hours after injection and disappeared within 6 hours.

2. *Red cell agglutination.* The blood of rats injected intravenously with doses up to 2.5 mg of poly-D L-lysine hydrochloride per 100 g bodyweight did not show red cell agglutination. Red cell agglutination was observed only in the heart blood of the rat dying after injection of 8 mg per 100 g bodyweight.

3. *Red blood cell and reticulocyte count.* The results are given in Table II. Single or repeated intravenous injections of 1-2 mg of poly-D, L-lysine hydrochloride per 100 g bodyweight (sublethal) caused a drop in the red cell count of 14 to 45% and a reticulocytosis ranging up to 16%. Single or repeated injections of 0.75 mg or less per 100 g bodyweight caused a variable drop in the red cell count. In 2 rats reticulocytosis without a fall in red cell level was observed. Peripheral smears showed no abnormality of the red cells. Bone marrow examinations in 2 rats revealed marked erythroid hyperplasia.

4. *Clinical and anatomical picture caused by toxic doses.* Rats injected intravenously with doses of 1.5 mg or more of poly-D L-lysine hydrochloride per 100 g body weight died within approximately 12 minutes. The larger the dose the earlier death occurred. In order of appearance the signs were: sneezing, irregular rapid respiration, cyanosis, increased movements, convulsions, frothy pink fluid issuing from the nose and death in flaccid paralysis. Doses of 1.2-1.5 mg of poly lysine hydrochloride per 100 g of body weight caused temporary dyspnea and slight cyanosis which disappeared within approximately three-quarters of an hour. The pertinent patho-

TABLE II. Fall in Red Cell Count and Reticulocytosis in Rats Receiving Poly-D L-Lysine Intravenously.

Poly-D L-lysine HCl, mg/100 g body wt, i.v.	Max fall in R.B.C.* % of initial value	Max fall in R.B.C.* On day	Max reticulocytosis %	Max reticulocytosis On day
1	24	3	9	5
1	17	3	7.4	6
1	14	6	5	6
1	20	3	7.4	2
.75†	0		0	
.5	0		0	
.25	0		0	
3 × 1.5 > 6 hr	40	3	12.8	5
2 × 1 ½ "	45	3	13	3
3 × 1 6 days	16	6	8	6
3 × 1 + 2 × 2 + 1.5 >8 days‡	31	9	16	11
3 × .75 > 4 hr	35	3	11	4
3 × .75 8 "	0		0	
2 × .75 24 "	0		0	
3 × .6 2 "	12	2	12.8	3
3 × .6 2 "	7	2	5.7	3
3 × .6 2 "	0		8	3
3 × .6 2 "	0		6.4	3

* Preinjection red cell counts done at least 3 times, within 3 to 6 days prior to inj., including day of inj. Only those rats used whose preinjection counts did not differ more than 600000 per cmm, *i.e.* approximately ± 3% from avg count.

† Rats not showing any change were counted at least 6 days.

‡ This rat did not die when receiving 2 mg/100 g body wt and apparently had become resistant.

logical findings were limited to the lungs and heart. The lungs of rats injected with lethal doses of poly-lysine were 2 to 3 times the normal size and weight, markedly distended, bright pink, and oozed a great deal of frothy pink fluid from the bronchial tree and cut surfaces. Microscopically the majority of the alveoli contained protein rich material, red cells or a mixture of these. Perivascular and peribronchial edema was marked. The heart was dilated predominantly on the right side and histologically there was evidence of cardiac edema in both left and right ventricles. No gross or microscopic findings in the other organs could be attributed to the polylysine. Rats were injected with sublethal doses of poly-lysine and were sacrificed 1½ to 3 hours after injection. Grossly the lungs of these animals were normal. Histologically a small number of alveoli contained hyalin eosinophilic membranes flattened against the alveolar septa. Slight to moderate perivascular and peribronchial edema was visible in the larger connective tissue septa. Nothing else of note was found in the remaining organs.

5. *Antagonistic action of heparin and poly-L-aspartic acid to poly-D L-lysine in vivo.* Heparin as well as synthetic poly-L-aspartic acid protected rats from the fatal effects of intravenous poly-D L-lysine in a weight ratio of 1:1 to 1.5:1,* either when mixed with the polylysine before injection or by intravenous administration not more than 3 minutes after injection of the poly-lysine. Histological examination of the organs of these rats showed no pathological changes. In only a few of several experiments however did the acidic polymers completely neutralize the disturbance in thrombin formation caused by poly-lysine. The *clot-delaying effect* of heparin *in vivo* could be neutralized by polylysine, either by mixing polylysine with it before injection or by intravenous administration of polylysine after injection of heparin. Effective acidic polymer to basic polymer ratio in all these experiments were 1:1 to 1.5:1. The drop in red cell count caused by polylysine could in some cases be prevented by mixing it with 1.5-2 times the amount of heparin before injection. However a slight reticulocytosis was observed sometimes even though

* Poly-L-aspartic acid injected in these quantities was not toxic, did not affect thrombin formation, nor did it cause red cell agglutination.

the red cell count did not fall.

Discussion. Some of the known *in vitro* actions of poly-D L-lysine, *i.e.* inhibition of thrombin formation(2), red cell agglutination (7,8) and red cell destruction(9), were also shown to occur in rats after intravenous administration. The *in vivo* action of polylysine on the blood coagulation mechanism manifested itself in disturbed prothrombin conversion while clotting times remained normal. The disturbance in thrombin formation might be attributed to antithromboplastic activity (2). Red cell agglutination did not occur following lethal and sublethal doses even when thrombin formation was disturbed. Only when a very high lethal dose of polylysine was given, the clotting time became prolonged and red cells became agglutinated. Red cell agglutination occurred apparently only after much of the polylysine was bound to the plasma proteins(8). Red cell destruction was probably due to direct damage to the red cell (9). The respiratory signs appearing after toxic doses of polylysine were probably due to pulmonary edema and hemorrhage, cardiac dilatation and edema. The mechanism of this has not been elucidated. No anatomical lesions were found in the central nervous systems to explain the neurological disturbances which might have been the result of sudden anoxia consequent to pulmonary and cardiac lesions.

The acidic polymers, heparin and poly-L-aspartic acid, were antagonistic to the lethal, hemolytic and coagulation disturbing effects of polylysine. Similar antagonistic actions were found *in vitro*(2,8,9).

Summary. Poly-D L-lysine was administered intravenously to rats. Lethal doses of 1.5-2 mg per 100 g body weight caused a disturbance in thrombin formation, pulmonary and cardiac edema and death within 12 minutes. The high dose of 8 mg polylysine per 100 g body weight caused red cell agglutination and prolonged clotting time. Sublethal doses also caused a disturbance in thrombin formation, slight hemolysis and reticulocytosis, and transitory symptoms of respiratory distress. Heparin and synthetic poly-L-aspartic acid were antagonistic to polylysine and prevented death of polylysine-treated rats, hemolysis and coagulation disturbance. These effects *in vivo* corroborated the observations on the biological action of polylysine *in vitro*.

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Intracellular Localization of Succinoxidase and Cytochrome Oxidase of the Kidney Cortex.* (20079)

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New concepts of intracellular localization have resulted from the chemical analysis of particles isolated by differential centrifugation of tissue homogenates. Thus, "large granules" from kidney isolated in alkaline water contain 66% of the total succinoxidase and cytochrome oxidase activity(1). Technics utilizing 0.88 M sucrose solution as the dispersion medium have since then been introduced(2) in order to preserve the morphologic integrity of mitochondria. The present study utilizes these newer methods in a complete fractionation of the renal cortex and the determination of these oxidative enzymes.

Experimental. Sherman strain rats weighing 175-225 g maintained on Purina chow, were used as a source of the material for the determination of the normal renal cortex partition. In the fasting experiments the weights ranged from 125-175 g. Animals were killed after the intraperitoneal injection of veterinary nembutal (0.1 ml/200 g) and the kidneys were rapidly removed and stripped of the perirenal fat, fascia, and capsule. The kidneys were blotted on filter paper to remove the extraneous blood, chilled, and the cortices separated from the medulla. The weight of this tissue was then obtained. The tissue was placed in an evaporating dish which was kept in an ice-bath. A quarter of each kidney was put in Zenkers solution for histologic examination. The cortical tissue was then cut into small pieces and transferred to a Potter-Elvehjem homogenizing tube. Homogenization of the tissue was done with a steel or glass grinder for 5 minutes, and 0.88 M sucrose solution added to the preparation so as to obtain a final volume 10× the wet weight of the tissue, so that the final concentration was 100 mg tissue per 1 ml of sucrose solution. All these procedures were performed in a cold room at temperature 1°-4°C. The total homogenate (H) was spun according to the procedure of Schneider, Hogeboom, and Palade(2). The first sediment (Pel 1) was removed at 600 × g for 2 minutes on an International Clinical Centrifuge and consisted of nuclei, unbroken cells, red blood cells and mitochondria (or "large granules"); the supernatant (Sup 1) consisted of mitochondria and microsomes but no nuclei, red cells or unbroken cells. This supernatant (Sup 1) was spun at 20,000 × g for 20 minutes and a tan-brown pellet (B-Pel) was obtained which contained mitochondria and some microsomes. This pellet was then washed a number of times with 0.88 M sucrose. The supernatant from which the pellet was obtained (Sup 2) was then centrifuged at 20,000 × g for 120 minutes at which time a red, translucent pellet was obtained which consisted of microsomes (R-Pel). This pellet was also washed repeatedly. The final supernatant (F-Sup) was a translucent reddish yellow fluid. All of the stages in the fractionation were followed with observations by phase microscopy. All fractions were brought to the volume of the original homogenate from which they were derived prior to removing aliquots for biochemical and microscopic analysis. Succinoxidase and cytochrome oxidase activities were determined manometrically(3). "Endogenous" cytochrome C was determined in a system wherein tissue, buffer, electrolytes but no cytochrome C were added. Nitrogen was determined according to the Ma-Zuzaga modification of the micro-Kjeldahl method(4).

Results. The succinoxidase, cytochrome oxidase and the "endogenous" cytochrome C of the renal cortex have been determined. All the results are reported in terms of 1.0 ml of the original 10% homogenate. In accordance with previous reports(1,2) the greatest per cent enzyme activity is present in the cytoplasm (Sup-1) and, within the cytoplasm, in

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the mitochondrial fraction (B-Pel). On the basis of wet weight, we find that 43% of the total succinoxidase activity and 49% of cytochrome oxidase activity is localized in the mitochondrial or "large granule" fraction of the renal cortex. On the basis of nitrogen, succinoxidase has an activity of 220 μl O_2/10′/mg N in the original homogenate and an activity of 497 μl O_2/10′/mg N in the mitochondria. The activity of cytochrome oxidase in the original homogenate and the mitochondria is 590 μl O_2/10′/mg N and 1656 μl O_2/10′/mg N respectively.

Discrepancies in recovery values of our findings with the earlier figures(1,2) led to a further investigation of the effects of repeated centrifugal washings on the mitochondrial fraction and the microsomal fraction. It was found (Table I) that in contrast to the reported lack of effect of repeated washings of the mitochondria of liver, mitochondria of the renal cortex lose a great deal of succinoxidase and cytochrome oxidase activity. After 2 washings there is a 58% decrease in succinoxidase activity per wet weight and 45% decrease in cytochrome oxidase activity per wet weight, whereas on the basis of nitrogen there was a 40% decrease in succinoxidase activity and no decrease in the cytochrome oxidase activity of the washed kidney mitochondria. These data show that succinoxidase activity decreases as rapidly as the nitrogen decreases while cytochrome oxidase activity decreases at a much slower rate than nitrogen after 2 washings.

In order to account for the relative decrease in enzymatic activity of the mitochondrial fraction noted after repeated washing, other fractions were combined with the mitochondria and the succinoxidase activity was determined (Table II). Although additions of Pel-1 and R-Pel resulted in no increase in succinoxidase activity, small amounts of the final supernatant fluid (F-Sup) raised the total activity of the mitochondria to 85% of the activity of the total homogenate. The succinoxidase activity of the added final supernatant fluid was so small that it contributed no inherent activity to the end result.

50% of the total "endogenous" cytochrome C of the cytoplasmic extract (Sup-1) was spun down with the mitochondria; whereas 40% of the "endogenous" cytochrome C of the total homogenate remained in the "nuclear" fraction (Pel-1). Since no unbroken cells were found this could indicate adsorption of "endogenous" cytochrome C to nuclear material. Previous determinations(5,6) report cytochrome C primarily localized in "large granules."

Differences in the effect of homogenization with the use of metal and ground glass homogenizers were noted. The nuclear fraction (Pel-1) accounted for 24% of the total succinoxidase activity or 134 μl O_2/10′/mg wet weight, when metal was used, and for 8% or 47 μl O_2/10′/mg, when ground glass was used. The difference can probably be accounted for by the greater number of unbroken cells present after metal grinding, a finding which, however could not be definitely confirmed by phase microscopy.

In accord with previous reports(7) there was little decrease in the activity of succin-

TABLE I. Effect of Washing on Succinoxidase Activity of Mitochondria.

Fraction	μl O_2/10′/mg wet	μl O_2/10′/mg N
B-Pel	241 (162–291)	497 (304–615)
" , W_1*	165 (115–210)	419 (361–489)
" , W_2*	99 (66–120)	300 (251–330)
" , W_3*	87 (78–100)	310 (300–317)
" , W_4*	88 (68– 94)	367 (350–410)

* W, with subscript, refers to No. of washings.

TABLE II. Effect on Succinoxidase of Adding Small Amounts of "Nuclei," Microsomes, and Final Supernatant Fluid to the Mitochondrial Fraction.*

Fraction	μl O_2/10′/mg wet	% of H
B-Pel	206	37
" + Pel-1	210	38
" + R-Pel	220	40
" + .04 ml F-Sup	320	57
" + .12 ml "	430	76
" + .24 ml "	480	85

* In all cases amounts added were so small as to contribute negligible inherent activity. Thus any increase in activity was due to some "activator" contained in the added fraction.

oxidase and cytochrome oxidase of the total homogenate or the fractions after 16-140 hours of starvation.

Discussion. These experiments indicate that much less of the total succinoxidase and cytochrome oxidase activities were recovered with the renal cortex mitochondria isolated in sucrose than in whole kidney mitochondria isolated in alkaline water(1). This may be due to differences between cortical and medullary mitochondria or to differences induced by the suspending medium. In addition, less succinoxidase activity can be demonstrated in the renal cortex mitochondria than in mitochondria from other tissues. Thus, although there is qualitatively a tissue and species biochemical similarity of mitochondria, quantitatively there may exist tissue and possibly cellular differences.

Summary. 1. The results with 0.88 M sucrose solution as the dispersion medium agree essentially with those obtained with alkaline water medium. 2. Succinoxidase activity of the mitochondria accounts for 43% of the total homogenate activity and cytochrome oxidase 47% on a wet weight basis. 3. On nitrogen basis the activities are 2-3 times greater than that of the homogenate. 4. After 2 washings the activity of both enzymes on a wet weight basis is reduced; on a nitrogen basis succinoxidase activity is reduced to 33% but the cytochrome oxidase activity is the same as in the original mitochondria. 5. Small amounts of the final supernatant fluid added to mitochondria increase the succinoxidase activity to 85% of the original homogenate. This effect does not represent an addition of the activities of the 2 fractions. 6. There is no change in intracellular succinoxidase and cytochrome oxidase activities after a period of starvation of 16 to 140 hours.

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Anti-Gonadal Hormone Activity of 11α-Hydroxyprogesterone. (20080)

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The antagonism which one gonadal hormone may show for another is frequently brought about by the hormones' decreasing the secretion of gonadotrophins by the pituitary, and, therefore, secondarily diminishing the production of the endogenous sex hormones. On the other hand, there is evidence that a gonadal hormone may inhibit the effects of another sex hormone directly. Early investigations by de Jongh(1-3) revealed that androgens are capable of preventing certain responses in estrogen-treated animals. These observations were confirmed and extended by Zuckerman and Parkes(4), Robson(5), Gardner and Pfeiffer(6) and many others. The anti-androgen effects of estrogens were reported about the same time by Gley and Delor(7) and Mühlbock(8). Early experiments by Macht and Stickels(9), Hisaw and Lendrum(10) and Dessau(11) brought out the inhibitory effect which progesterone may exert on the biological activity of estrogen. Conversely, estrogen has been shown to be antagonistic to the development of certain progestational effects. The inhibition of the placentoma reaction by estrogen has been demonstrated by Courrier(12), Brouha(13), Votquenne(14) and others. Although both estrogen and progesterone are

needed for the production of the progestational type of endometrium, an excess of estrogen prevents this uterine response(15-17). There are numerous other reports of antagonism between gonadal hormones. The antagonism which one gonadal hormone has for another under certain conditions has led to their use in clinical medicine in attempts to counteract sex hormone-linked pathology. Although this therapy has proven successful in some instances, the effectiveness of a steroid in antagonizing the endogenous hormone is frequently vitiated by its own sex hormone activity. It is reasonable to assume that the efficacy of this technic would be enhanced if a steroid were available which exhibited antigonadal hormone activity without having other pharmacological activity.

A number of 11-oxygenated compounds related to the steroidal hormones have recently become available through new processes developed in the laboratories of The Upjohn Company(18-20). As part of a broad program of biological evaluation of these steroids, we have investigated the endocrinological behavior of 11α-hydroxyprogesterone (U-0384), a compound differing from progesterone in that it has an α-hydroxyl group at position 11 of the steroid nucleus. This new steroid has been found to antagonize estrogen and androgen while having little or no other hormone-like effects.

Procedures. U-0384 was tested for antigonadal hormone activity in estrogen-treated gonadectomized male and female rats and in androgen-treated castrated male rats as well as in intact untreated male and female rats. The compound was also assayed for estrogenic, androgenic, progestational, and adrenal cortical hormone activity. The animals used in these procedures were rats of the Upjohn Strain which are of Sprague-Dawley ancestry. At the beginning of the experiments the rats were divided into groups of equal body weight. The various steroids which were used were homogenized and suspended in a diluent prepared in this laboratory (0.4% Tween 80, 1.5% benzyl alcohol, 0.9% NaCl, and 0.5% low viscosity carboxy-methyl cellulose in distilled water). The experimental rats were injected subcutaneously once a day with the steroid suspended in 0.4 cc of the diluent; control animals were given the diluent alone. In procedures where U-0384 and a gonadal hormone were administered simultaneously to the animals, the two steroids were injected at different sites.

Anti-estrogen effect in ovariectomized female rats. Fifteen immature female rats with an average body weight of 63 g were ovariectomized and divided into 3 groups. The rats were injected daily with diluent, 0.05 μg estradiol (17β-estradiol), or 0.05 μg estradiol and 10 mg of U-0384. The animals were treated for 4 days and killed 24 hours after the last injection. The uterine weight of the rats injected with estrogen plus U-0384 was 76 mg as compared with 111 mg in the estrogen-treated rats and 53 mg in the controls. This experiment has been repeated several times with substantially the same results. It is concluded that U-0384 partially counteracted the stimulation of the uterus in ovariectomized rats injected with estradiol. *Anti-estrogen effect in castrated male rats.* Fifteen immature male rats averaging 78 g were castrated and divided into 3 groups. The rats in the first group were injected with 0.4 cc of the diluent. The second group was given 20 μg of estradiol and 0.4 cc of the diluent, and the rats in the third group were injected with 20 μg of estradiol and 10 mg of U-0384. The animals were treated for 5 days and killed 24 hours after the last injection. The seminal vesicles of the estrogen-treated rats weighed 26.4 mg as compared with 7.0 in the uninjected controls. Since the administration of U-0384 to estrogen-treated rats reduced the seminal vesicle weight to 18.6 mg, it appears that this steroid partially counteracted the exogenous estrogen. This experiment has been repeated under varying conditions and essentially the same results obtained.

Anti-estrogen effect in intact immature female rats. Twenty-five immature female rats of the Upjohn strain were divided into 5 groups. The rats in the first group were injected with the diluent. The rats in the remaining groups were given 5 or 10 mg of progesterone or U-0384. The animals were treated for 10 days and autopsied 24 hours after the last injection. The data presented

TABLE I. Anti-Estrogen Activity of U-0384 in Intact Immature Female Rats.

Treatment (mg/day)	Body wt, g	Absolute wt (mg) Ovaries	Uterus	Thymus	Adrenals
Diluent	120	25.3	156	423	34.6
P* 5	127	16.1	123	383	27.8
P 10	122	13.2	117	366	25.1
U 5	121	20.5	88	390	28.4
U 10	121	19.6	96	404	31.4

* P = Progesterone; U = U-0384.

TABLE II. Anti-Estrogen Effect of U-0384 Compared with Testosterone in Intact Female Rats.

Treatment	Uterus (mg)	Ovaries (mg)	Body wt (g)	No. rats
Diluent	154	27.3	123	15
TP,* 500 μg/day	254	19.7	131	10
U-0384, 5 mg/day	81	18.4	119	15

* TP = Testosterone propionate.

in Table I demonstrate that U-0384 caused considerable atrophy of the uterus. Although progesterone was more effective in inhibiting the pituitary secretion of gonadotrophic hormones by ovarian weight criteria, the uteri of the animals were much heavier than in the rats treated with U-0384. This indicates that U-0384 is much more effective than progesterone in antagonizing endogenous estrogen. *Anti-estrogen effect compared with an androgen in intact female rats.* To compare the anti-estrogen effect of U-0384 with androgen, immature female rats were divided into 3 groups. The animals were injected for 10 days with diluent, testosterone propionate, or U-0384. On the eleventh day the rats were killed and the ovarian and uterine weights taken. The data from this experiment appear in Table II. The secretion of gonadotrophic hormones was suppressed by each of the steroids injected as evidenced by the decreased ovarian weights. U-0384 was effective in causing uterine atrophy whereas the androgen caused further stimulation of uterine growth. It was considered unreasonable to give the testosterone at the same dosage as the U-0384. The dose given is very large and probably comparable to any amount used clinically in women. The increase in body weight of the rats getting the androgen confirms that this amount of testosterone is more than adequate to produce the anabolic effect. Although androgens under some conditions counteract estrogens, in this experiment the testosterone brought about an enlarged uterus whereas U-0384 effectively antagonized the endogenous gonadal hormones.

Anti-androgen effect in castrated male rats. Fifteen young male rats were castrated and divided into 3 groups. The rats in the first group were injected with the diluent, and the second group was given 200 μg testosterone propionate. The animals in the third group were injected with 200 μg testosterone propionate and 10 mg of U-0384. The animals were treated for 3 weeks and autopsied 24 hours after the last injection. The results of the experiment are recorded in Table III. The purpose of this experiment was not only to test the activity of U-0384 in decreasing the androgen-induced growth of the sex accessories, but also upon the nitrogen-retention effect of androgen as measured by the weight of the body, kidney, and the levator ani muscle. From the data in Table III it is seen that U-0384 partially counteracted the androgen-induced hypertrophy of the seminal vesicles, prostate and levator ani muscle. This experiment has been repeated several times with essentially the same results. *Anti-androgen effect in intact male rats as compared with estrogens.* To compare the anti-androgen activity of U-0384 with estrogen, adult male rats were divided into 4 groups, 5 rats to the group. The rats were injected for 10 days with diluent, estradiol, stilbestrol, or U-0384. On the eleventh day the animals were killed and the weights of the seminal vesicles, prostates, and testes were taken. The data from this experiment are in Table IV. Contrary to expectation, the dose of estradiol was not enough to cause great atrophy of the secondary sex organs. The stilbestrol, however, was injected in amounts which were sufficient to elicit marked involution. It should be emphasized that the doses of estrogen used were sufficient to produce great uterine stimulation in females. The decrease in body weights obtained with the estrogens indicates that the doses were excessive. Based on the weight of the seminal

TABLE III. Anti-Androgen Activity of U-0384 in Castrated Male Rats.

Treatment	Body wt (g)	Absolute wt in mg			
		Sem. ves.	Prost.	Kidney	Lev. Ani.
Diluent	188	11.5	17.4	1870	39.8
TP,* 200 μg/day and diluent	204	214.8	276.2	1943	158.6
TP, 200 μg/day, and U-0384, 10 mg/day	179	163.4	211.6	1990	109.6

* TP = Testosterone propionate.

TABLE IV.
Anti-Androgen Activity of U-0384 as Compared with Estrogens in Intact Male Rats.

Treatment	Body wt (g)	Sem. ves. (mg)	Prostate (mg)	Testes (g)
Diluent	247	199	196	2.634
Estradiol, 5 μg/day	231	125	110	2.469
Stilbestrol, 50 μg/day	199	46	44	2.110
U-0384, 5 mg/day	251	118	147	2.430

vesicles (which is better criterion of androgen activity than prostate weight) U-0384 was about as effective as 5 μg of estradiol in antagonizing the androgen. All of the compounds produced some testicular atrophy. While U-0384 caused atrophy of the seminal vesicles, it is not estrogenic and caused no decrease in body weight.

Estrogenic activity. Twenty immature female rats were ovariectomized and divided into 4 groups. The animals were injected for 8 days with diluent, or U-0384, in a daily dose of 1, 5, or 10 mg. U-0384 had no estrogen-like activity as determined by uterine stimulation: on the contrary, there was some decrease in uterine weight at the larger doses. The uterine weight of rats given 10 mg of U-0384/day was 43 mg as compared with a weight of 57 mg in the untreated controls. It is postulated that this was due to the anti-estrogen effect of this compound. Since the rats were ovariectomized, it is presumed that there was a small amount of estrogen being produced by the adrenals(21,22).

Androgen-like activity. Twenty male rats were castrated and divided into 4 groups. The rats in the first group were injected with the diluent, and the rats in the remaining groups were given 1, 5, or 10 mg of U-0384. The animals were treated for 10 days and autopsied 24 hours after the last injection. The weight of the seminal vesicles and prostates of the untreated rats averaged 12.0 and 16.4 mg respectively. Since the corresponding weights in rats treated with U-0384 in doses as high as 10 mg/day were 11.7 and 18.7, this steroid has little or no androgenic activity.

Progestational activity. The method chosen for testing U-0384 for progestational activity was that described by Astwood(23), based on the fact that a progestin is required for the formation and maintenance of the deciduoma in the traumatized uterus of the pseudopregnant rat. Adult female rats of the Holtzman-Rolfsmayer strain, 200-220 g body weight, were mated with vasectomized males to induce pseudopregnancy. On the fourth day after mating, the rats were ovariectomized, and the left horn of the uterus was traumatized by scarification of the endometrium with a needle. Beginning on the day of operation and continuing for 3 days, the rats were injected once daily with A) progesterone, 0.25 or 0.50 mg, or B) U-0384, 1.0 or 3.0 mg. On the following day following the last injection the rats were sacrificed, the uteri were dissected out, and mean diameters of control (right) and traumatized (left) horns of the uteri were estimated with a millimeter rule. It was concluded that since a positive response was obtained with 0.25 mg of progesterone, and since negative responses were obtained with 3.0 mg of U-0384, this compound has less than 1/12 the progestational activity of progesterone.

Effect on gonadotrophic hormone secretion.

Although U-0384 did not have estrogenic, androgenic, or progestational activity in the doses tested, it is of interest that this steroid caused a moderate suppression of gonadotrophic hormone secretion based on ovarian atrophy (Tables I, II). In the preceding experiments in which U-0384 had an anti-gonadal hormone effect in intact animals, it is probable that the action was both by direct antagonism to the sex hormone, and via an inhibitory action on the secretion of gonadotrophic hormone.

Effect on the secretion of ACTH. In Table I data are presented from intact female rats given U-0384 in doses of 5 and 10 mg/day. Using relatively large amounts there was no evidence of thymolysis, although there was some suppression of the secretion of ACTH as evidenced by slight adrenal atrophy. U-0384, however, was less active in this regard than comparable amounts of progesterone. U-0384 was also administered to gonadectomized male and female rats in doses of 5 and 10 mg/day without producing a decrease in body or thymic weight, although again there was slight adrenal involution. Since this compound had very little effect on the adrenal weight and no effect on the thymic and body weights of rats given very large amounts, it is probable that the steroid has little or no adrenal cortical hormone activity.

Summary. 1. U-0384 (11α-hydroxyprogesterone) partially counteracted the effects of exogenous estradiol in stimulating the uteri of ovariectomized rats and the seminal vesicles of castrated male rats. This compound also decreased the hypertrophy of the seminal vesicles, prostate, and levator ani muscle in castrated males injected with testosterone propionate. In intact rats U-0384 decreased the weight of the sex accessories by its anti-gonadal hormone activity, and through its inhibitory effect on gonadotrophic hormone secretion. 2. This steroid has no estrogenic, androgenic, or progestational activity in the amounts assayed. This compound, when injected in large amounts, produced only slight adrenal atrophy and no thymic involution, and can, therefore, be presumed to have very little or no adrenal cortical hormone activity. 3. Although the anti-estrogen and anti-androgen activity of this new steroid is limited in animals, inasmuch as it lacks other gonadal hormone effects, it may be effective in therapy of some pathological conditions in which a reduction of endogenous sex hormones is considered desirable.

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Mobility of Skeletal Phosphorus in a Mature Dairy Cow as Determined with Radioactive Phosphorus.* (20081)

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The use of radioactive tracers to study skeletal mineral metabolism has resulted in a number of significant findings. It has been shown that the skeleton is not a stable structural tissue but continuously exchanges minerals with the tissue fluids of the body and is a very important reservoir of Ca and P. Several early workers, among them Manly and Bale(1), recognized that the skeleton was not a homogeneous system with respect to the behavior of its minerals but actually consisted of 2 fractions. One fraction of the skeleton they found in rapid exchange with the minerals of the plasma and they called this fraction "labile." The remaining minerals were held firmly by the skeleton and were slowly interchanged with plasma minerals only when apatite crystals were formed and resorbed. These minerals represented the "stable" fraction of the bone.

Several methods, *in vivo* and *in vitro*, have been used to estimate the size of this "labile" pool in bones and to determine its role in mineral exchange between the plasma and the skeleton. The estimates for the size of the labile calcium pool range from 15%(2) to 20%(4) of the total bone calcium. The labile phosphorus pool has been estimated to be 20%(3), 13%(5), and 17%(1) of the total bone phosphorus using different experimental methods in each case with small animals. Unless the magnitude of the "labile" exchange process is known it is difficult to interpret results obtained with radioactive tracers in studies of mineral metabolism. This report deals with the determination of the "labile" pool in the mature dairy cow and with an investigation of the problem of sampling skeletons of large size in isotope studies.

Experimental. An aged (10 years) lactating dairy cow weighing 354 kg was injected intravenously with 13.9 millicuries of phosphorus-32 and sacrificed 72 hours later by electrocution.‡ Bones were removed as rapidly as possible (all within 1 hr), scraped free of most adhering tissue, and placed in a boiling water bath for 5-10 minutes. This treatment simplified the removal of all remaining soft tissue from the bones. The bones were air-dried for 48 hours, weighed, and then samples of them were taken for analysis. Long bones were cut in half at the center of the shaft and then split lengthwise so that the marrow could be removed. Samples of cortical bone were obtained by filing across the cut surface of the shaft and samples of trabecular bone by scooping a small quantity out of the pocket at the ends of the long bones. Composite samples were collected as sawdust after sawing through the longest axis of the bone. For the flat bones of the skull, pelvic girdle, rib and scapula, holes were drilled at random through the bones and the borings collected for the composite sample of each bone. Each sample was dried at 105°C for 24 hours to determine the percentage dry matter and then ashed at 550°C until a white powder was obtained. The ash was dissolved in a few ml of 6N HCl and diluted to a known volume. Aliquots of this solution were assayed for phosphorus-32 by the method described by Kleiber(8). The total phosphorus of these same solutions was determined by the method of Fiske and SubbaRow(9). *Autoradiograms.* Bone sections from the desired location were cut as thin as possible with a fine bladed coping saw. These sections were further reduced in thickness by rubbing

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‡ After sacrifice the cow was found to have a fetus about 4 months old. The effect of this fetus on the phosphorus uptake of the maternal tissues is not known at present.

TABLE I. Specific Activity of Bone Samples from Different Parts of the Skeleton of a Mature Dairy Cow.

Sample	Stand. spec. activity, $\mu c/g$ P / mcP^{32} inj./kg B.W.	Spec. activity ratio (P^{32}/P^{31}) in skeleton / (P^{32}/P^{31}) in inorganic fraction
Composite bone sample		
3rd tail bone (1.6 g)	9.2	.66
9th " " (7.3 g)	19.6	1.4
Carpal	6.0	.43
Tarsal	12.0	.86
Metacarpal	8.9	.64
Metatarsal	9.3	.67
Radio-ulna	18.9	1.36
Humerus	11.0	.79
Tibia	16.6	1.2
Femur	16.3	1.17
Pelvic girdle (ilium)	11.0	.79
" " (pubis)	14.1	1.0
Skull	12.4	.89
Scapula (blade)	10.7	.77
" (distal end)	18.8	1.35
Rib (7th)	15.6	1.12
Vertebra (6th lumbar)	21.2†	1.53
" (9th thoracic)	25.6	1.84
" (3rd cervical)	50	3.6
Cortical bone		
Tibia shaft	7.2	.52
Femur shaft	8.7	.63
Radio-ulna shaft	7.8	.56
Humerus shaft	9.1	.65
Humerus (exterior surface of epiphysis)*	9.2	.66
		Avg = .6
Trabecular bone‡		
Tibia (distal end)	22.5	1.62
" (proximal end)	24	1.72
Femur	23.2	1.67
Radio-ulna	25.6	1.84
Humerus	20.8	1.5
Vertebra (6th lumbar)	16.1†	1.16
		Avg = 1.7
Marrow		
Tibia	256	18.4

* This sample of bone was filed from the outer surface of the proximal end of the humerus and sufficiently shallow so that no trabecular bone is included.

† We believe these samples may have been interchanged during processing which would account for the higher specific activity found in the composite sample as compared to the trabecular bone sample.

‡ Spongy cancellous bone found in the epiphysis of long bones and throughout the interior of flat bones and vertebra.

against sand paper of decreasing coarseness and finally on jeweler's rouge paper to obtain a smooth glossy surface. This surface was placed in direct contact with a piece of Ansco Triple S Pan film in a light-tight box and exposed for a predetermined period of time. The exposure period was determined by the time required for 8 to 10 million beta particles per sq cm to emerge from the bone surface in contact with the film. This exposure gave satisfactory contrast between regions of different phosphorus-32 concentration.

Results. The specific activity of each bone sample assayed is listed in Table I. In the first column the specific activity (microcuries P^{32}/g phosphorus) is expressed per unit of relative injected dose (millicurie phosphorus-32 injected per kg body weight). The advantage of expressing results in this manner (standard specific activity) arises when one compares data from this experiment with data obtained under similar conditions from animals of different body size and those which have received a different quantity of phosphorus-32. The second column in Table I lists the specific activity of each bone sample relative to the specific activity present in the inorganic fraction of the plasma. This expression reveals the extent to which the phosphorus of the bone has come into isotope equilibrium with the inorganic phosphorus of the plasma.

There is a large variation of the standard specific activities of the composite bone samples depending on the anatomical origin of the bone. In general, those bones in or near the vertebral column have the greatest P^{32} uptake and the small peripheral bones of the leg and foot the lowest P^{32} uptake. The large leg bones and the flat bones of the pelvic girdle, rib, skull, and scapula have an intermediate phosphorus turnover as indicated by their specific activities. The same variation of isotope uptake for different components of the skeleton has also been observed in rat (6,7,10) and human skeletons(11). This variation in phosphorus exchange for bones from different anatomical locations complicates skeletal metabolism studies in large animals because it is impractical to get an aliquot sample of the entire skeleton and individual bones must be used.

The sampling problem is further complicated by the variation in P^{32} uptake found in different anatomical regions of a single bone.

The specific activity of the cortex from the tibia shaft had 1/3 the specific activity of trabecular tissue from the epiphysis of the same bone. This variation is also found in samples taken from similar regions in other bones and confirms results obtained by several workers with other animals. Harrison(12) followed calcium-45 uptake by rats and found the specific activity 2 to 3 times greater in the epiphysis than the diaphysis 48 to 72 hours following the administration of isotope. Neuman(5) made an *in vitro* study of exchangeable P and found it to be 3 times as great in the epiphysis as in the diaphysis of fresh bone. Similar variations in phosphorus-32 uptake by different regions of the bone have been reported in skeletal studies of dogs(13), rabbits (14) and rats(15).

In small animals it is possible to radioassay the complete skeleton for mineral turnover studies but in the cow the size of the skeleton makes this an impractical approach and it is even difficult to obtain an aliquot sample of any single bone except the smallest foot and tail bones. The preliminary problem, then, was to establish a suitable method for obtaining a sample of the bones of the cow that would represent the average skeletal mineral turnover but would not require grinding up of the whole skeleton.

The specific activities of cortical bone from several different anatomical regions (Table I) are nearly constant. The trabecular bone samples also have rather constant specific activities but some 300% higher than that found for cortical bone. Each of the composite samples has a specific activity intermediate between the cortical and trabecular bone samples.§ This result suggests that the variation of specific activity observed for different skeletal components results from differences in the mass ratio of trabecular/cortical bone in different anatomical regions. The high specific activity always observed for bones in the vertebral column which have a large mass ratio of trabecular/cortical bone and the lower specific activity of peripheral bones that have a lower mass ratio of trabecular/cortical bone substantiates this relationship. The epiphysial region of long bones where trabecular bone is concentrated generally has a specific activity 2 to 3 times greater than the diaphysis(5,12-15) which contains mainly cortical bone. The specific P^{32} activity of cortical bone scraped from the exterior surface of the humeral epiphysis was the same as cortical bone from the shaft of the humerus (Table I) suggesting again that the controlling factor in P^{32} uptake is primarily the structural type of bone (trabecular or cortical) involved rather than anatomical location.

As a result of these observations it was decided that the most satisfactory method for sampling skeletal tissue in the cow would result from radioassaying a few samples of cortical bone from the shafts of long bones and trabecular bone from the epiphysis of long bones and using the average value for each. These average values establish the limits within which the specific activity for the skeleton as a whole should fall and by estimating the mass ratio of trabecular/cortical bone in the skeleton one may obtain an approximate value for phosphorus turnover.

The total weight of the skeleton for this cow was 29 kg‖ and the average phosphorus content of the bones sampled was about 10%, thus giving a total skeleton content of 2.9 kg phosphorus. Assuming that the skeleton is 60% cortical bone and 40% trabecular bone, one would conclude that the cow has 1.7 kg phosphorus in cortical bone (specific activity = 0.325 μc P^{32}/gP) and 1.2 kg phosphorus in trabecular bone (specific activity = 0.91 μc P^{32}/gP) which gives a total of 1.6 millicuries of P^{32} or 11.6% of the injected isotope in the skeletal tissue.

The relative specific activities listed in the second column of Table I show the extent to which bone phosphorus has equilibrated with plasma inorganic phosphorus and provide the data needed to calculate the size of the

§ The 3rd cervical vertebra is an exception and this may have been due to traces of marrow tissue which are difficult to remove completely from vertebral bones.

‖ Some of the small foot bones were not included. Weight of ribs based on weight of 7th rib and total weight of each spinal section was based on the vertebra recovered.

"labile" phosphorus pool. It cannot be stated unequivocally that only the "labile" pool of the bone contains phosphorus-32 though it is necessary to make this assumption to calculate the size of the "labile" pool. However, studies that have been made on the 2 skeletal pools all indicate that the process of recrystallization is very slow compared with the surface exchange process(1,5,6,16). In addition, Neuman(16) produced evidence suggesting a decrease in the rate of recrystallization with increasing age of the animal. A 10-year-old cow is physiologically very old and thus recrystallization would be expected to constitute a negligible factor in P^{32} uptake by the skeleton during the 72-hour interval of the experiment. The "labile" fraction of the skeleton, on the other hand, is very rapidly exchanging with plasma minerals as indicated by the rapid skeletal deposition of isotopes in pigs as early as 2½ minutes after injection(4). The specific activity of the plasma for our cow decreased very slowly after 3 hours indicating that a state of near-equilibrium exists between the plasma and those pools rapidly exchanging phosphorus with the plasma. Data obtained from rat bones(5) indicate that an equilibrium state between the plasma and the bone labile phosphorus pool has been established within 2 days. The assumption is justified, therefore, that an equilibrium state has been established at 72 hours between the "labile" phosphorus pool of the skeleton and the plasma which means that the specific activity in each pool is the same. The measured specific activity of the bone will differ from that of the plasma due to the diluting effect of the stable bone which is not exchanging phosphorus and therefore the specific activity of the bone relative to that of the plasma (specific activity ratio—Column II, Table I) directly expresses the percent of the bone phosphorus in the labile fraction.

The specific activity for cortical bone is 0.6% and that for trabecular bone 1.7% of the plasma specific activity which means that the "labile" phosphorus pool is about 3 times more extensive in trabecular bone than cortical bone. The size of the "labile" phosphorus pool for the whole skeleton would be between 0.6% and 1.7% and for a mass ratio of 40% trabecular/60% cortical the value would be 1%. This "labile" pool would therefore amount to 29 g (1% of 2.9 kg phosphorus in total skeleton) of phosphorus.

The plasma contained 8.4 mg % inorganic phosphorus. In a total volume of 22.6 liters of plasma (determined with Evans Blue) this gives a circulating pool of 1.9 g phosphorus. The labile phosphorus pool in the skeleton therefore contains a phosphorus store some 15 times greater than that present in the circulating plasma pool.

It is not readily apparent why the labile pool constitutes only a small fraction (1%) of the skeletal phosphorus in the cow when values as high as 17%(1) to 20%(3) have been obtained for rat skeletal tissue. This difference between labile pool sizes does not arise from our assumption that recrystallization played a negligible part in P^{32} uptake. If this were a factor then the calculated value of 1% labile P in the cow would be too large and the discrepancy between cow and rat skeletal tissue would be even greater. The explanation may be that the tissue mass of the cow requires a relatively greater quantity of cortical bone for support and that this results in less vascularity for the skeletal tissue of the cow and therefore less surface for exchanging phosphorus with tissue fluids.

The autoradiograms in Fig. 1 and 2 give a picture of the anatomical distribution of the labile phosphorus pool. B, Fig. 1, is an autoradiogram of the smallest bone at the distal end of the tail. The marked deposit of P^{32}

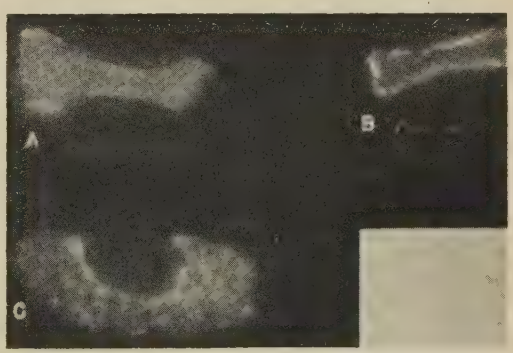

FIG. 1. Autoradiograms showing distribution of P^{32} in cow bones. A and B are longitudinal sections of tail bones and C is a cross section of half of the tibia shaft. Note the discrete deposition of P^{32} which occurs in cortical bone as represented in C.

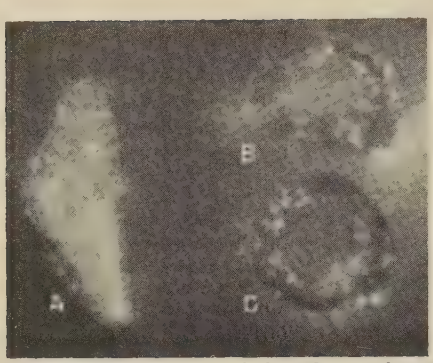

FIG. 2. Autoradiograms of foot bones from the cow. (A) a tarsal bone, (B) metacarpal; (C) metatarsal. Note the more uniform deposition of P^{32} in trabecular bone (central area in A) as compared to the discrete deposition in cortical bone (B, C, and outer area in A).

in this anatomically remote bone indicates that the labile phosphorus pool must extend through every part of the skeleton. This bone has no trabecular region and the P^{32} is all present in the outer cortical bone. The larger tail bone (A, Fig. 1) contains trabecular areas and shows a correspondingly greater P^{32} content inside the bone. The labile pool in cortical bone as illustrated by the cross section through the tibia shaft (C, Fig. 1) exists in discrete deposits and is apparently associated with the Haversian system that permeates bony tissue. This relationship would be expected since the small blood vessels of the bone pass along the Haversian canals and the "labile" pool must maintain intimate contact with these blood vessels to permit rapid mineral exchange.

Fig. 2 shows the distribution of this labile phosphorus pool in 3 bones of the foot, one of which, a tarsal bone (A), contains extensive trabecular bone. The general distribution of the labile pool throughout the trabecular region as opposed to the discrete distribution of this pool in cortical bone is apparent in these autoradiograms. This difference in distribution of the labile pool probably results from differences in the nature of the blood supply to these 2 types of bone. In cortical bone only the phosphorus along the Haversian canals has sufficient contact with the blood supply for rapid exchange while the less dense trabecular bone has a much larger surface in contact with the tissue fluids and thus a greater quantity of phosphorus may exchange per unit of time.

Summary. 1. Radioactive P^{32} was injected intravenously to measure the "labile" phosphorus pool in the skeleton of a mature, lactating dairy cow. This pool contains about 1% of the total skeletal phosphorus which was 15 times as large as the total circulating phosphorus pool in the plasma. 2. Autoradiograms and radioassays indicated that this labile pool extended throughout the skeleton but its distribution was determined by the type of bone involved. Cortical bone contains only one-third as much phosphorus in the labile state as does trabecular bone. 3. About 12% of the injected phosphorus-32 was present in the skeleton at the time of slaughter of the animal, 3 days after the injection.

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Improved Knife-Holders for Thin-sectioning with Rotary Microtomes.* (20082)

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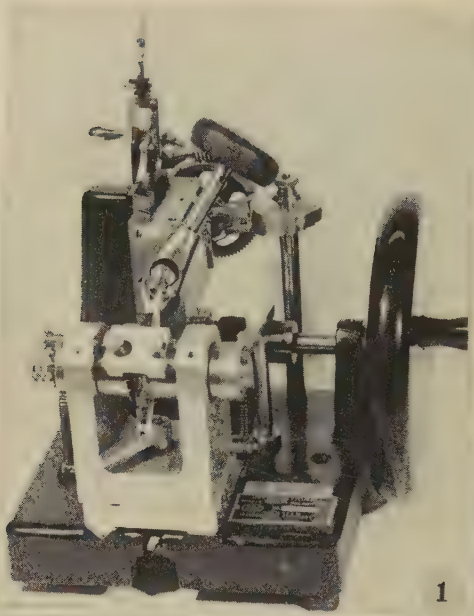
Various adaptations of ordinary microtomy have been proposed to permit cutting thin sections of tissue for electron microscopy. Pease and Baker(1) described an auxiliary wedge for the Spencer microtome which reduced its advance by one-tenth, and reported that double-imbedded tissues could be sectioned at adequate thinness. Latta and Hartman(2) found that glass knives were preferable to the usual steel microtome knife. Imbedding in *n*-butyl methacrylate has proved the present medium of choice (Newman, Borysko and Swerdlow(3); Palade(4)). The Minot rotary microtome has been modified by the introduction of a reduction gear into its drive so that advances of 0.05 μ per revolution are possible, and measurements of the sections secured with this instrument have indicated they approximate this thickness (Geren and McCulloch)(5). Some experience with the Spencer and Minot (International) thin-sectioning microtomes convinced us that adequate sections were routinely possible only if cut onto a water meniscus, as proposed by Gettner and Hillier(6). However, a major difficulty with the meniscus arose upon the return stroke of the microtome. When the specimen passed the knife edge, the meniscus touched the block and the section, previously cut, adhered to the block and was lifted off the meniscus. Serial ribbons, therefore, could not be cut. Moreover, the greatest variations in thickness occurred when the first few sections were cut after a period of inactivity; thus, the time consumed between sections by the necessary removal and mounting of the sections one at a time became a major concern.

These difficulties were overcome by mounting the knife holder upon ball-bearings so that the knife-edge was rotated away from the block on the up-stroke of the microtome. A cam and lever arrangement, illustrated in Fig. 1-4, accomplishes this motion automatically. The ratios chosen are such as to move the knife edge backwards approximately one-half millimeter. Since any play in the bearings or looseness in the knife-holder assembly would cause chatter marks or inequalities in the sections, care was taken to clamp rigidly all portions of the assembly. Return of the knife-edge to exactly the same point during the cutting cycle is accomplished by loading the bearings with strong springs. With these precautions, chatter marks and thick areas in the section are eliminated.

The sections, when cut, are floated upon 30% acetone. Minor wrinkles and compressions are rectified by the surface action of this drop. If viewed with reflected light through a low power dissecting microscope, the flattening of the sections can easily be observed, and the interference colors produced in the thin sections can be used as a rough but adequate index of their thickness. The sections are mounted directly upon collodion-coated grids of copper mesh. The grid, held in fine forceps, is sunk beneath the surface of the acetone solution, moved under the desired sections and lifted together with them out of the water. When done with the aid of a low-power binocular, positioning of the section upon the grid is possible.

Recently, Hillier(7) has described a technic for sharpening steel knives for thin sectioning. Some experience by one of us indicated advantages of the long cutting edges on steel knives and the larger water reservoir which can be constructed for them (Lansing and Hillier)(8). However, ribbons of sections are not always secured, for the same reasons outlined above for the glass knife. Consequently, a steel knife holder was designed incorporating the rocking mechanism (Fig. 3 and 4). This knife holder is interchangeable with those for the glass knives.

* This investigation was supported in part by a research grant from the National Institutes of Health, Public Health Service and the Damon Runyon Memorial Fund.



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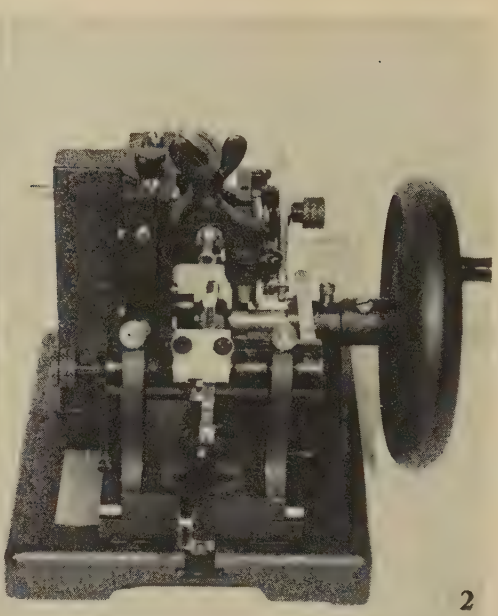

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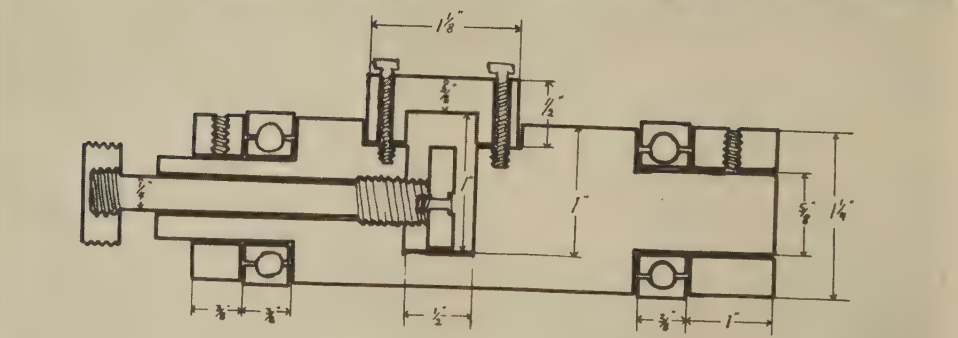

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FIG. 1. Minot rotary microtome, adapted for thin-sectioning by the International Equipment Company and modified by the addition of the rocking holder for glass knives described in this article. The cam and lever arrangement, and the adjustable stop to control the excursion of the knife edge are shown.

FIG. 2. Rotary microtome manufactured by Bausch and Lomb, modified for thin sectioning by introducing a worm and pinion gear into the drive mechanism and by adding the rocking holder for glass knives.

FIG. 3. Holder assemblies for glass knives (left) and steel knives (right). On the glass knife, a water-trough is constructed by building up a dam of paraffin. For the steel knife, a

metal trough is employed, sealed to the knife blade by a rubber gasket. The larger metal trough permits longer ribbons of sections to be prepared.

FIG. 4. Scale drawing of the glass knife holder illustrated above. The material is brass except for the ball bearings which are available commercially.

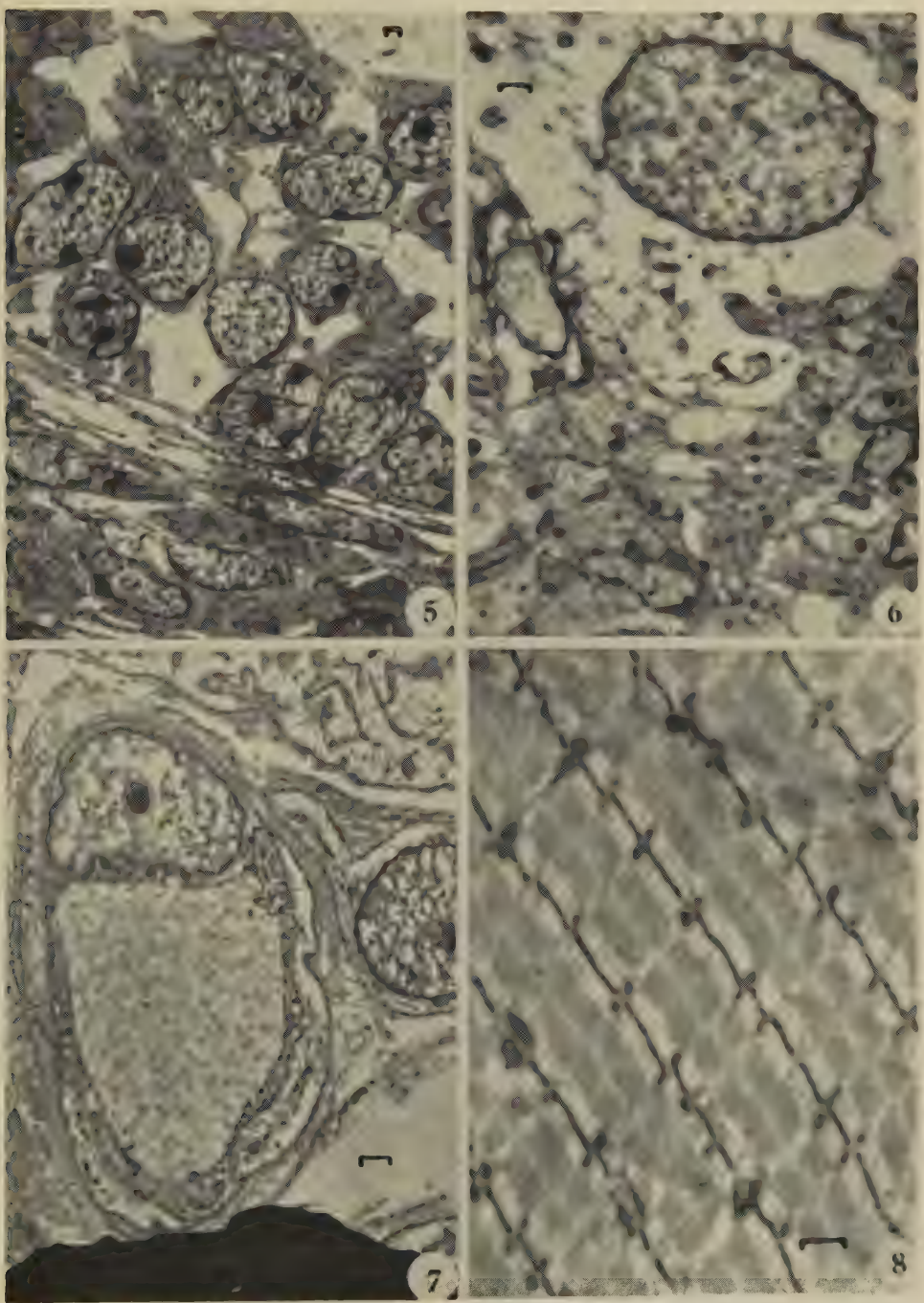

FIG. 5. Electron micrograph of a section through an ovarian follicle in a 5-day-old guinea pig. Liquor folliculi is shown in the upper right corner, granulosa cells occupy the middle portion of the field, and thecal cells are at the bottom. This section was cut with a glass knife.

FIG. 6. Electron micrograph through the spinal cord of a new-born mouse. In the upper portion, a cell and its nucleus are present. The lower portion of the picture represents cell-processes and fibers. The age of the mouse accounts for the fact that little myelin is seen. This section was cut with a steel knife.

FIG. 7. Electron micrograph of a capillary from the tongue of a cat. The section was cut with a glass knife.

FIG. 8. Electron micrograph of a section through the gastrocnemius muscle of a mouse. The section was cut with a glass knife.

All sections illustrated on this plate were from tissue fixed by Palade's buffered osmic acid procedure and imbedded in butyl and methyl methacrylate mixed in a ratio of 3:1. The black line on each photograph represents one micron.

In practice, adequate sections have been secured with either knife. Longer ribbons can be cut with the steel knife. On the other hand, glass knives are quickly interchangeable and our impression is that with the microtome advances available the thinnest sections possible are more readily cut with the glass knives.

A Minot microtome, manufactured several years ago by the Bausch and Lomb Co. was also modified for thin sectioning. A reduced rate of advance was obtained by introducing a worm and pinion gear with a ratio of 100:1 into the advance mechanism. The knife holder was also modified as described above. Fig. 2 illustrates the arrangement of the various components in this instrument.

Both microtomes, modified as described, have given good service. The instrument manufactured originally by the International Company routinely cuts ribbons at thicknesses of 0.05 μ and above. The Bausch and Lomb instrument similarly produces ribbons, but in this case the lower limit of thickness is 0.025 μ. For most purposes sections approximately 0.1 μ thick are adequate. However, some structures encountered in sections of mammalian tissues are quite dense and the thinner sections offer a great advantage. Epidermis, hair and nail, for example, require very thin preparations.

Fig. 5-8 are electron micrographs of sections cut with these instruments. They illustrate the uniformity of thickness which can be obtained, the freedom from chatter marks, and the adequacy of the sections for resolving detail without its being obscured by overlying structures.

Summary. A modification of the knife holder for thin-sectioning microtomes has been described. A cam and lever arrangement, timed with the cutting cycle, advances the knife edge to the cutting position on the down stroke of the instrument and withdraws the knife edge approximately one-half mm on the return stroke. This modification permits the routine cutting of serial ribbons of uniform thinness. The ribbons are flattened upon the surface of an acetone-water meniscus and can be lifted directly on to suitably coated wire grids for electron microscopy. Sections as thin as 0.025 μ have been prepared routinely.

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Manometric Studies on Oxidation of Choline by Avian Liver Homogenates.* (20083)

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The presence in chick liver of an enzyme system oxidizing choline has been reported (1). The importance of this enzyme system has become evident in recent years in that reports have appeared indicating that choline may have to be oxidized to betaine before its methyl groups can become available to the organism for transmethylation reactions(2, 3). Williams *et al.*(4) have developed an assay method for the determination of choline oxidase in rat liver homogenates. This method, however, has limited usefulness in studies with chick tissues. The endogenous oxygen uptake for the chick liver homogenate is relatively high and the chick liver homogenate is quite sensitive to the influence of tonicity. The present report concerns the study of factors which influence the oxidation of choline by homogenates and washed suspensions of chick liver.

Experimental. Unless otherwise indicated, New Hampshire chicks of 8 to 10 weeks of age, fed a commercial type broiler ration,† were used. In the preliminary experiments, the method of Williams *et al.*(4) was used to measure the choline oxidase activity of chick liver homogenate. All enzyme activity was assayed in a Warburg bath at 37°. This method utilizes a system containing 1.0 ml of the 16.7% homogenate, 1.0 ml of distilled water, and 10 mg of choline chloride. Endogenous respiration is measured in a similar system containing no added choline. The center well contained 0.2 ml of 10% KOH in all flasks. The water and choline chloride were added from the side arm at the end of a 10-minute equilibration period. Since the oxygen uptake was maximum during the first 20 minutes and then dropped rapidly, the oxygen uptake during this period was employed as a measure of choline oxidase activity. All enzyme activity is reported on the basis of fresh liver weight.

Results. Factors influencing oxidation of choline by homogenates of chick liver. 10 mg choline chloride per flask was the optimum concentration Contrary to the results of Williams *et al.*(4) the addition of 17.5 mg niacinamide per flask did not affect the endogenous oxygen uptake or choline oxidase activity of chick liver homogenate. The addition of betaine increased the endogenous oxygen uptake but this was probably a tonicity effect since 16 mg KCl could also produce a similar increase. The addition of 22 mg betaine to choline chloride depressed by 28% the choline oxidase activity. Therefore, the procedure for the determination of choline oxidase in chick liver homogenate was modified to eliminate the high endogenous respiration and the influence of tonicity.

Factors affecting oxidation of choline by washed suspension of chick liver. A 16.7% homogenate of chick liver was prepared in isotonic KCl solution (1.13%) containing 0.04% $NaHCO_3$ with the Potter-Elvehjem glass homogenizer, and this was centrifuged at 1700 x *g* for 10 minutes at 5°C. The supernatant was found to have less than 5% of total choline oxidase activity and was thus discarded. This finding is in accord with earlier reports(5,6). The residue was thoroughly mixed with 4 ml of ice cold isotonic KCl solution and recentrifuged as above. This operation was repeated 3 times, finally the residue being resuspended in enough isotonic KCl to give a suspension equivalent to a 33.3% homogenate. Chick liver suspension prepared according to this procedure was found to give only 10 to 15% of the endogenous O_2 uptake of that noted with an equivalent amount of the whole homogenate. Addition of niacinamide and betaine and further addition of KCl had no

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† Texas A & M Poultry Farm Stock Ration.

effect on endogenous O_2 uptake or choline oxidase activity.

The influence of choline chloride concentration on the O_2 uptake during the 20-minute period was studied with a suspension equivalent to 333 mg fresh tissue. The values obtained were as follows: 2 mg, 47 μl of O_2; 10 mg, 60 μl; 20 mg, 77 μl; and 50 mg, 40 μl. Thus 20 mg of choline chloride in 2 ml was optimal. A straight line was obtained by using a substrate concentration ranging from 0.2 mg-2 mg of choline chloride and plotting the reciprocal of choline oxidase activity against the reciprocal of the substrate concentration. Michaelis constants for 4 separate experiments were 2.0, 2.0, 2.1 and 1.7 x 10^{-3} M, which are within the range of those reported by Eadie and Bernheim(7) using rat liver suspensions.

With the washed preparation no significant differences were obtained in the oxygen uptake with pH values ranging from 6.8-7.6. At pH 7.3 liver suspensions gave a maximum O_2 uptake in the first 20-minute period and then gradually decreased with time. Therefore, pH 7.3 was employed for routine determinations of choline oxidase in chick liver suspensions. Cytochrome *C* did not increase the oxygen uptake under these conditions.

Washed suspensions of liver were prepared in isotonic KCl solution and in isotonic sucrose solution (0.25 M). In both cases centrifugations were done at 9500 x *g*. In 4 different experiments, 5 to 10% more O_2 uptake was obtained with suspensions of liver made in isotonic KCl in comparison to the one in isotonic sucrose. To determine the influence of centrifugal force on the choline oxidase activity, 2 liver suspensions were prepared with 2 different centrifugal forces, one at 1700 x *g* and another at 9500 x *g*. The choline oxidase values for the 2 liver suspensions using 20 mg choline chloride were 79 and 80 μl of O_2 per 20 minutes respectively. 1700 x *g* was therefore used for routine determinations.

TABLE I. Choline Oxidase Activity of Avian Tissue Suspensions.

Preparation	Choline oxidase, μl O_2/20 min.
Chick liver	85
" kidney	74
Turkey liver	110
" kidney	81

Flask components: 1 ml tissue suspension equivalent to a 33.3% homogenate in isotonic KCl solution along with .5 ml of .08 M sodium-potassium phosphate buffer (pH 7.3) and .2 ml of 10% choline chloride solution. Water was added to a total vol of 2 ml. Two-tenths ml of 10% KOH was placed in center wells. Prior to closing stopcocks, flasks were equilibrated for 10 min. at 37°C.

Determinations of choline oxidase were made on liver and kidney suspensions of chick (New Hampshire), turkey (Beltsville small white) and turtle (Chelydra serpentina) using the same procedure. The results are shown in Table I. Turtle liver and kidney were devoid of any detectable choline oxidase activity.

Summary. Factors influencing the manometric determination of choline oxidase activity in chick liver homogenate and washed liver suspension have been studied and a procedure is outlined for the measurement of this enzyme system in washed suspensions.

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Purification and Physiological Properties of Factor VII from Plasma and Serum. Separation from Prothrombin. (20084)

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The existence of Factor VII was first recognized in serum*(1). A one-stage method for the quantitative determination of this factor has been proposed(1); it yields practically the same results with plasma or serum.† Separation of Factor VII from prothrombin offers considerable difficulties, although the physiological behavior of these two clotting factors are entirely different: Factor VII accelerates conversion of prothrombin to thrombin; prothrombin, on the other hand, determines quantity of thrombin formed. The two factors can be partly separated: a) by the clotting process itself, more than 95% of prothrombin being consumed, whereas Factor VII remains unchanged in the serum; b) by dicumarol and similar anticoagulants which cause a more rapid decrease of Factor VII-concentration at the beginning of therapy. The difficulty of the chemical separation of Factor VII and prothrombin is a consequence of the similarity of their physico-chemical properties. No separation can be obtained by fractional precipitation with different salts, organic solvents, or their combined use. Fractional adsorption on barium sulfate, tricalcium phosphate, etc., also is ineffective. The relative amounts of the two factors remain unchanged after such procedures. Only by means of *chromatography* is it possible to isolate prothrombin and Factor VII. Prothrombin and Factor VII are separated from fibrinogen and Factor V by adsorption on barium sulfate, the mixture prothrombin-Factor VII is then chromatographed. By this chromatographic procedure, first pure prothrombin and later pure Factor VII are obtained.

Method. Purification of Factor VII from plasma. The first two steps are carried out at 2°C, the third and fourth at room temperature. *Step 1. Adsorption.* 10 g of barium sulfate (Merck feinst gepulvert, Germany) are suspended in 200 cc of oxalated plasma. The suspension is stirred for 40 min., then centrifuged. The supernatant, containing fibrinogen and Factor V, is discarded. *Step 2. Washing.* The barium sulfate is washed twice with 30 cc of physiological saline. The solution is discarded. *Step 3. Preparation of column for chromatography.* A homogenous mixture of barium sulfate (10 g) and hyflo-supercel (5 g) suspended in physiological saline is introduced into the column. The excess of saline is again discarded. *Step 4. Elution.* The prothrombin is eluted by means of 150 cc of .14 molar trisodium citrate-citric acid solution at pH 5.8. The elution is continued with a .14 molar trisodium citrate solution at pH 7.8. After addition of the latter, the pH of the eluate increases slowly until a final value of 7.8 is attained. First prothrombin is eluted, then a mixture of prothrombin and Factor VII, and finally Factor

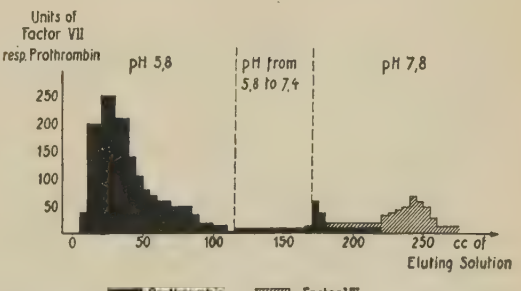

FIG. 1. Chromatographic separation of prothrombin and Factor VII. Eluting solutions: 1) Trisodiumcitrate-citric acid .14 m, pH 5.8. 2) Trisodiumcitrate .14 m, pH 7.8. Eluate fractions of 5 cc.

* Factor VII is almost certainly identical with SPCA of Alexander *et al.*(2), co-thromboplastin of Mann and Hurn(3), proconvertin of Owren(4), stable prothrombin conversion factor of Owen and Bollmann(5), and the accelerator factor of MacMillan(6).

† In undiluted plasma and serum, results obtained by the one-stage method are somewhat different; this is due to a difference in the prothrombin content of plasma and serum. After dilution (1:10) results of Factor VII assay are identical in plasma and serum.

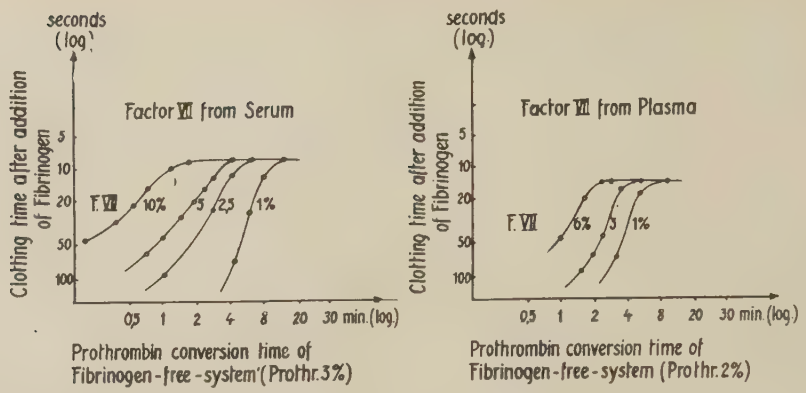

FIG. 2. Acceleration of thrombin formation by increasing amounts of Factor VII. Clotting system: .1 cc prothrombin-Factor V sol., .1 cc Factor VII sol. (variable), .1 cc thromboplastin, .1 cc $CaCl_2$ 1/40 m. Incubation of this mixture = prothrombin conversion time (abscissa). Ordinate, clotting time after addition of fibrinogen (.1 cc of 500 mg % sol.) is plotted in reverse order so that direction of the arrow signifies increasing amounts of thrombin.

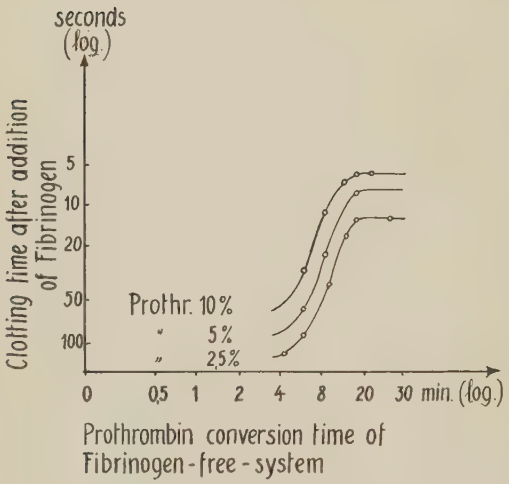

FIG. 3. Increase of quantity of thrombin formed by increasing amounts of prothrombin. Clotting system (see Fig. 2). In this system the Factor VII is constant, the prothrombin variable.

VII free from other clotting factors. The elution is performed under pressure in order to assure a sufficient speed of flow (one drop/12 sec.).

Purification of Factor VII from serum. Because of the minute amount of prothrombin in normal serum the purification of Factor VII offers fewer difficulties in serum than in plasma. All the operations are carried out between 2 and 5°C(7). *Step 1.* Factor VII is adsorbed on barium sulfate (Röntgen, Merck, Germany). 20 g of barium sulfate are suspended in 200 cc of diluted serum (9 parts of serum, 1 part of 0.1 M sodium oxalate). The suspension is stirred 45 min., then centrifuged. *Step 2.* The supernatant is discarded. The barium sulfate is washed twice with 30 cc of physiological saline and once with 30 cc of a .006 M sodium citrate solution. *Step 3.* Factor VII is eluted by .14 M solution of sodium citrate (pH 7.8). The barium sulfate is suspended in 30 cc of citrate, stirred 30 min., then centrifuged. The supernatant solution contains Factor VII (yield 90%) and can be used for the study of Factor VII.

Control of activity. The one-stage methods for the determination of prothrombin and Factor VII have previously been reported(1).

Results. The chromatographic separation of Factor VII and prothrombin from plasma results in clotting factors physiologically pure, *i.e.*, free of other coagulation factors (Fig. 1). The final prothrombin yield is comparatively high, 50% or more, whereas Factor VII recovery is very low and never exceeds 10%.

The effect of prothrombin and Factor VII from serum on the clotting process have previously been differentiated(1). Comparison of physiological properties of Factor VII obtained from plasma and from serum is now possible. Both preparations give the same acceleration effect on thrombin formation (Fig. 2). Prothrombin, on the other hand, prepared by the chromatographic method (Fig. 1) has no effect on velocity of thrombin formation; its concentration is proportional to the amount

of thrombin formed (Fig. 3). The curves of Fig. 2 and 3 were obtained in a fibrinogen-free system, all the clotting factors being kept constant except the one to be studied (Factor VII in Fig. 2, prothrombin in Fig. 3). The thrombin formed is determined by addition of fibrinogen. The identity in the mode of action of Factor VII from plasma and serum is obvious. Fig. 3 shows that the amount of thrombin formed depends on the quantity of prothrombin in the system. As already mentioned(1) the one-stage method for quantitative determination of Factor VII (using Seitz-filtered Factor VII-free oxalated ox-plasma) yields the same results with material obtained from plasma or serum.

Chemical and physical comparison of the two proteins is not yet possible, the quantity of the products being insufficient.

Summary. Methods of purification of Factor VII from plasma and serum are outlined. The separation of Factor VII and prothrombin from plasma is accomplished by means of chromatography. The mode of action of Factor VII from plasma and serum proved to be identical.

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Response of Young Dogs to West Nile Virus. (20085)

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The virus employed in this study was the B956 strain of West Nile virus furnished by the American Type Culture Collection in Washington, D. C. This virus was obtained by the above institution from Dr. K. C. Smithburn(1) of the Rockefeller Foundation Laboratories in New York. He had isolated this virus strain from a patient in Uganda, Africa and had passed it 25 times in Swiss albino mice by intracerebral inoculation. This virus has been cultivated in chick embryos(2) and in baby chicks(3). The virus, on receipt at this laboratory, was in the form of lyophilized mouse brain of the 25th passage. Three-weeks-old Swiss albino mice (Webster strain) were employed. This strain of mice was the one recommended by Webster(4) as being especially susceptible to neurotropic viruses. The dogs used were mongrels 5 to 6 weeks of age and averaged 4 to 5 lbs each.

The lyophilized mouse brain of the 25th passage was diluted to a 10% suspension with physiological saline. Each of 6 mice was inoculated intracerebrally with 0.03 cc of this 10% suspension. The mice showed involuntary motor reactions on the 3rd day post inoculation. When these symptoms of central nervous system involvement appeared, the mice were sacrificed, and the brains were removed aseptically. The pool of infected brains was ground with alundum and diluted to a 10% suspension with physiological saline. The suspension was then subjected to 5 minutes centrifugation in a horizontal centrifuge at 2,000 r.p.m. The supernatant was used as the inoculum for initiating the virus experiment in the dogs. The virus titrated 10^{-5} in Swiss albino mice inoculated intracerebrally. The supernatant material was injected intracerebrally into 4 unvaccinated mice and into 4 mice which had been immunized previously with the 25th mouse passage

TABLE I. Response of Puppies to West Nile Virus of the 26th Intracerebral Mouse Passage, 2 Pups in each Experiment.

Route of inoculation	No. puppies showing symptoms	Incubation period (days)
Intracerebral	2	3, 4
Intranasal	1	5, 6
Intradermal	0	
Intracardiac	1	5, 6
Intraperitoneal	0	

of West Nile virus. Immunization was accomplished by intramuscular injections using high dilutions of the virus over a period of several weeks. After 3 days the unvaccinated mice showed symptoms of central nervous system involvement, while the immunized mice showed no nervous symptoms during a 21-day observation period. This confirmed the virus to be West Nile virus.

Ten healthy mongrel puppies were divided into 5 groups of 2 puppies each. Each puppy was given a 10 cc prophylactic dose of distemper antiserum in the morning and was treated with the West Nile virus in the afternoon of the same day. Puppies exposed intracerebrally or intranasally received 1 cc of the 10% suspension of West Nile virus, while the puppies exposed dermally, intraperitoneally or intracardially received 2 cc of this 10% virus suspension. After inoculation all groups were observed twice daily for the appearance of characteristic nervous symptoms. The results of this group exposure are given in Table I.

When symptoms of central nervous system involvement appeared, affected puppies were sacrificed and the brains removed aseptically. The puppy brains were ground separately with alundum and diluted to 10% suspensions with physiological saline. The suspensions were then subjected to 5 minutes centrifugation in a horizontal centrifuge at 2,000 r.p.m. The supernatant from each suspension was injected intracerebrally into 4 unvaccinated mice and into 4 mice which previously had been immunized with the 25th mouse passage of West Nile virus. After 5 days the unvaccinated mice showed symptoms of central nervous system involvement while the immunized mice showed no nervous symptoms during a 21-day observation period. This confirmed the virus in the brain material from each puppy showing West Nile symptoms to be West Nile virus. All puppies showing no West Nile symptoms were sacrificed at the end of a 14-day observation period. Brains were removed aseptically, ground separately with alundum, and diluted to 10% suspensions with physiological saline. The suspensions were then subjected to 5 minutes centrifugation in a horizontal centrifuge at 2,000 r.p.m. The supernatant from each suspension was injected intracerebrally into 6 3-week-old Swiss albino mice. The mice showed no nervous symptoms and were discarded after a 14-day observation period.

Summary. A strain of West Nile virus isolated by Dr. K. C. Smithburn from a patient's serum in Africa and passaged intracerebrally in Swiss albino mice has been successfully transmitted intracerebrally, intranasally, and intracardially to mongrel puppies.

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Virulence in Mice of Colonial Variants of *Candida albicans.* (20086)

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A previous note(1) on the chemotherapeutic activity of 6-sulfanilamido-2,4-dimethylpyrimidine (Elkosin), reported that this sulfonamide exerted a high degree of effectiveness against infections in mice induced by a number of micro-organisms, including *Candida albicans*. Based on 2 separate tests made with the latter organism, Elkosin was found to be active whereas sulfadiazine and sulfisoxazole exhibited no activity. The results of the earlier tests have since been confirmed and extended in many subsequent experiments with the exception that we have been unable to reproduce the activity of Elkosin in *C. albicans* infections. This discrepancy between the earlier and later trials could have been caused by a number of factors. Several workers(2-4) have clearly shown that cultures of *C. albicans* undergo spontaneous variation particularly when maintained for long periods under laboratory conditions. George and Plunkett(4) found that of 10 cultures maintained for several years on Sabouraud's glucose agar, 6 strains exhibited varying degrees of colonial dissociation proceeding from the smooth, moist type to the rough, dry form with the development of a much-branched "bushy" mycelium in contrast to the compound verticils of blastospores observed in the original, freshly isolated cultures.

In order to determine whether such colonial variations result in an alteration in virulence or could have influenced the results of our chemotherapeutic assays with Elkosin, we have investigated, in addition to the strain (culture No. 300) used for the earlier chemotherapy tests, 2 other *C. albicans* strains maintained in our culture collection.

1. *Cultural characteristics.* Two of these strains were composed of colonial variants consisting primarily of either yeast forms (smooth, colony type) or pseudomycelial elements (rough, colony type). The smooth type was present in 2 variations with respect to the gross aspect of the colonial growth, one variant producing cream-colored, flat colonies and the other yielding similar colonies but with deeper, yellow centers. *Table I* describes the colonial and cell morphologies of these variants. In Sabouraud's dextrose broth after 24 hours of incubation at 37°C, all strains grew compactly at the bottom except for Strain No. 238 which produced a cottony-like type of growth in the lower third of the broth.

2. *Virulence studies.* The different variants were grown and prepared according to a modification of Strauss and Kligman's method (5). The yeast phase of a culture less than 10 days old was transferred to Sabouraud's broth and incubated with constant agitation at 37°C for 24 hours. After centrifuging and noting the volume of packed cells, the organ-

TABLE I. Morphological Characteristics of *C. albicans* Colonial Variants.

Culture	Colony characteristics on Sabouraud's agar (24 hr at 37°C)	Wet-mount microscopic appearance of cells
#300 S*	Flat, cream-colored smooth colonies	Many budding blastospores in small clusters and chains. Very few branching pseudomycelial cells.
#238 R	White, round, rough colonies with domed raspberry-like surface	Many long-branching pseudomycelial cells with attached clusters of blastospores and chlamydospores. Few free blastospores.
#285 S	Flat, cream-colored smooth colonies with yellowish centers	Many budding blastospores in pairs, chains and clusters. Very few branching pseudomycelial cells.
#285 R	Small, white, rough colonies with domed raspberry-like surface	Many elongated and oval budding blastospores in pairs, chains and rosettes.

* S = Smooth type; R = Rough type.

TABLE II. Mouse Virulence of Colonial Variants of *Candida albicans*.

Culture	Conc. of packed organisms	No. of mice	Survival time (days) Avg	Range
#300 S*	1:10	16	5.1	<1 – >18
	1:50	10	2.8	<1 – 11
	1:100	12	4.4	<1 – >18
	1:500	11	7.8	2 – >18
	1:1000	6	8.2	2 – 12
#238 R	1:10	11	6.6	<1 – >18
	1:50	5	7.8	2 – >18
	1:100	11	16.5	2 – >18
	1:500	11	18.8	17 – >18
	1:1000	11	19.0	>18
#285 S	1:10	11	1.1	< 1
	1:50	10	4.6	<1 – >18
	1:100	8	2.1	<1 – 3
	1:500	8	6.5	2 – >18
	1:1000	8	16.2	4 – >18
#285 R	1:10	13	4.0	<1 – >18
	1:50	10	1.5	< 1
	1:100	8	9.6	<1 – >18
	1:500	8	9.1	2 – >18
	1:1000	8	12.6	2 – >18

* S = Smooth type; R = Rough type.

isms were resuspended at varying concentrations in Sabouraud's broth containing 4% gastric mucin. Male CFW mice were infected intraperitoneally with 0.5 ml amounts of the different concentrations of culture and observed for a total of 18 days. As seen from *Table II* the results of these experiments suggest that the different colonial types possess varying degrees of virulence for mice. This is indicated by a comparison of the rough and smooth types with regard to the average survival times for the different culture dose levels. In this connection, it is of interest to note that George and Plunkett(4) found no differences in fermentative properties among the dissociated strains.

3. *Chemotherapeutic studies.* All variants were used for chemotherapeutic assays. Elkosin did not influence the infections induced by any of our strains of *C. albicans*.

Summary. 1. Several variants isolated from 3 cultures of *C. albicans* exhibited differences with regard to colonial morphology, microscopic appearance as well as virulence. The smooth colonial type of strain No. 300 proved to be more virulent than the rough colonial form of Strain No. 238. 2. The results of the previous chemotherapeutic tests with Elkosin in *C. albicans* infections could not be reproduced in tests performed with the newly isolated variants.

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An Activator System in Blood Indispensable for Formation of Plasmin by Streptokinase.*† (20087)

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The fibrinolytic agent produced by certain streptococci (streptokinase) was originally thought to be a fibrinolytic enzyme(1). Milstone(2) showed that in addition to the streptococcal factor, a "lytic factor" in serum was necessary for the production of fibrinolysis. Later, Christensen(3) found that plasminogen, a precursor in blood, was transformed to the enzyme, plasmin, by streptokinase. Streptokinase is considered a direct activator of plasminogen. Discrepancies have been ob-

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served, when plasminogen preparations from different species were activated by streptokinase and by tissue activators(4-6). These observations and the demonstration of a plasminogen activator in spontaneously active human blood(7) prompted a reinvestigation of the action of streptokinase on the fibrinolytic enzyme system in blood.

Methods and materials. Fibrinolytic activity was estimated by: *1. The standard fibrin method*(8) (the substrate contains a large amount of plasminogen). *2. The heated fibrin plate method*(9) (plasminogen in the substrate is destroyed by heating). *3. Casein digestion*(10) (3% neutralized casein, "Hammarsten," in borate buffer. The transmission at 270 mμ of the perchloric acid precipitated solutions was determined by a Hilger spectrophotometer. Under the applied conditions the extinction was directly proportional to the plasmin concentration). *Human and bovine globulin* were precipitated isoelectrically from serum(2) and redissolved to 1/2 (human) and 1/1 (bovine) of the original volumes. *"Spontane activator."* Globulin containing a large amount of plasminogen activator was prepared from spontaneously active human plasma(7). *Streptokinase activated human globulin.* 10 ml normal human serum was precipitated isoelectrically and dissolved to 2 ml in borate buffer containing 5 mg streptokinase. *Streptokinase.* "Varidase" (Lederle). *Bovine plasminogen.* A lyophilized preparation was prepared from bovine fibrinogen(7). *Buffers.* Diethyl barbiturate buffer (Michaelis) M/10 (fibrin plate experiments). Borate buffer M/20 (casein experiments). Both: pH 7.75. Sodium chloride added to ionic strength 0.15.

Results. Activity of streptokinase activated human globulin. The fibrinolytic activity of streptokinase activated human globulin was estimated by means of the 2 fibrin plate methods. A large effect was always found on standard fibrin plates (containing plasminogen) while the effect on heated fibrin plates (no plasminogen) was very low (Fig. 1). A number of other proteases (trypsin, chymotrypsin, spontaneously active bovine plasmin, a *B. subtilis* protease and an aspergillus protease) showed only slightly different effects on the two fibrin substrates(9). These results

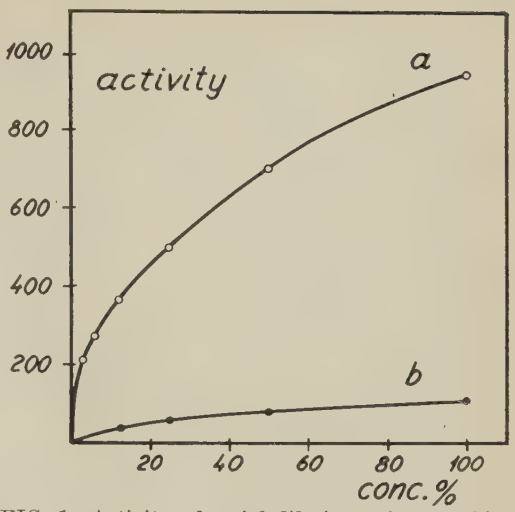


FIG. 1. Activity of serial dilutions of streptokinase activated human globulin measured on (a) standard fibrin plates (containing plasminogen) and (b) heated fibrin plates (no plasminogen). *Abscissa:* Concentrations in % of stock solutions. *Ordinate:* Activity as products of 2 diameters (mm^2) of the lyzed zones.

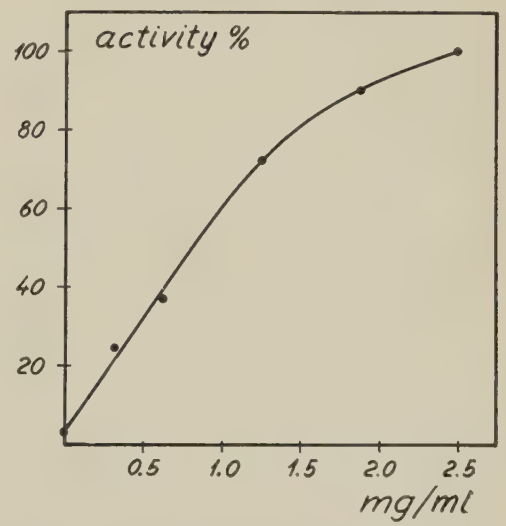


FIG. 2. Activity of mixtures of human globulin (dil. 1:60) and streptokinase (.006 mg/ml) with increasing concentrations of bovine plasminogen. *Substrate:* Heated fibrin plates. *Abscissa:* mg bovine plasminogen per ml. *Ordinate:* Activities as percentages of the maximal activity. (These values were obtained by interpolation of dilution curves prepared from each mixture(8). *Activity of controls:* Bovine plasminogen (2.5 mg/ml) + streptokinase (.006 mg/ml): 13%. Human globulin (dil. 1:60) + streptokinase (.006 mg/ml): 3%.

indicated that the large effect observed on standard fibrin plates was caused by an inter-

action of the mixture of human globulin and streptokinase with the bovine plasminogen contained in the substrate.

Effect of plasminogen. A mixture of streptokinase and human globulin was incubated with increasing concentrations of bovine plasminogen and the activity of the solutions was estimated on heated fibrin plates (Fig. 2). The activities were found to increase with the plasminogen concentration. Low activities only were produced, when solutions of human globulin and of bovine plasminogen were activated separately with streptokinase. These results suggested that the bovine plasminogen was activated by an agent formed by the interaction of streptokinase and human globulin.

Comparison of the activation of human and of bovine globulin. Bovine plasminogen preparations can be activated by tissue activators to much higher activities than those obtained by activation with streptokinase(4,5). Recently an excess of a plasminogen activator was demonstrated in spontaneously active human blood ("spontane activator")(7). These observations suggested the following explanation of our results. Streptokinase produces only a partial activation of the plasminogen in the bovine preparations. A complete activation of the bovine plasminogen is produced by an excess of activator formed by streptokinase in human globulin. This assumption was verified as follows. Solutions of human and of bovine globulin were activated by means of the "spontane activator." The resulting activities were compared with the maximal activities which could be produced by activation of the solutions by streptokinase. Heated fibrin plates were used (Table I). The "spontane activator" yielded the expected complete activation of all solutions. Twice as much plasmin could be produced by activation of bovine globulin with "spontane activator" as with streptokinase. Human globulin was activated to identical activities in both cases. The partial conversion of the bovine plasminogen by streptokinase was made complete when human globulin was added. Our bovine plasminogen yielded still smaller amounts of plasmin as compared with the high activities produced by addition of the "spontane activator."

TABLE I. Activation of Bovine and Human Globulin with "Spontane Activator" and with Streptokinase.

Mixtures	Measured activities of dilutions: 100	50	25	12.5%	Activities as % of maximal activity
B* .50 ml H .50 S 1.00	148	115	84	61	100
B .50 ml H .50 St 1.00	148	104	81	64	100
B .50 ml S .50 Bu 1.00	120	94	64	(33)	60
B .50 ml St .50 Bu 1.00	91	68	50	36	30
H .50 ml S .50 Bu 1.00	100	76	54	42	37
H .50 ml St .50 Bu 1.00	97	75	59	44	37

* B = Bovine globulin; H = Human globulin; S = "Spontane activator"; St = Streptokinase; Bu = Buffer.

Substrate: Heated fibrin plates. *Measured activities:* Products of two diameters of the lyzed zones (avg of 3 determinations) in mm². *Activities calculated as % of the maximal activity* were obtained by interpolation on the curves drawn from the measured activities(8). *Controls:* "Spontane activator," streptokinase, bovine globulin: inactive. Human globulin: slightly active (25 mm²). (The conc. of streptokinase and of "spontane activator" necessary to yield optimal activation of the mixtures were determined in a separate experiment).

The reaction between activator and plasminogen. In the following experiments bovine plasminogen was used as plasminogen and streptokinase activated human globulin as plasminogen activator. The *reaction time* (at 37°C) was estimated as follows. From a mixture of plasminogen and activator aliquots were withdrawn after various lengths of time and mixed with equal volumes of a casein solution. The digestion was followed by determination of the transmission at 270 mμ of the acid precipitated solutions (Fig. 3). The reaction was complete after an incubation period of 45 min., and a linear relationship between the extinction and the digestion time was found. The *nature of the reaction* was studied as follows. Plasminogen in varying concentrations was incubated with a constant amount of activator. Three series with 3 dif-

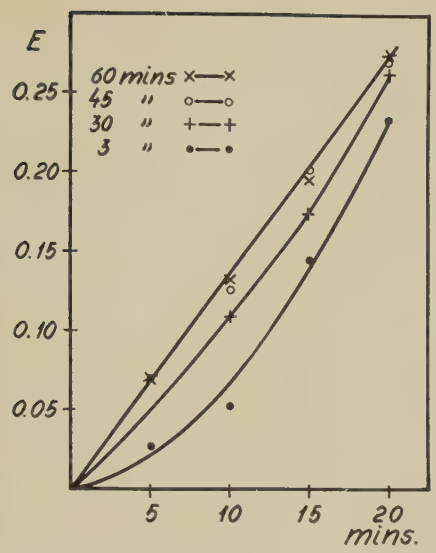


FIG. 3. Digestion of casein: Mixtures of bovine plasminogen (10 mg/ml) and streptokinase activated human globulin (dil. 1:100) were allowed to react for 3, 30, 45 and 60 min. Then equal volumes of the casein solution and of the mixtures were mixed and left at 37°C. After digesting for 5, 10, 15, 20 min., 2 ml aliquots were precipitated with 3 ml 10% perchloric acid. *Abscissa:* Digestion time. *Ordinate:* Extinction at 270 $m\mu$ measured with casein-buffer as reference and corrected for blank solutions.

ferent concentrations of activator were performed. The mixtures were allowed to react for 60 min. at 37°C. Casein was added and the transmissions of the acid precipitated solutions were estimated after digestion for 20 min. (Fig. 4). The curves show that the amount of plasmin produced depends: *1. In the case of an excess of activator,* on the plasminogen concentration; *2. In the case of an excess of plasminogen,* on the activator concentration.

This experiment indicates a stoicheiometrical mode of reaction between activator and plasminogen. It excludes the possibility of an autocatalytic reaction and of a catalytic action of human plasmin on bovine plasminogen. However, the instability of the activator in dilute solutions and the presence of an inhibitor in the plasminogen preparations complicate the quantitative evaluation of these preliminary experiments.

Discussion. The data presented here lead to the following conclusions: A. In preparations of bovine plasminogen streptokinase (in excess) produces low activities, which are smaller than those caused by other activators. B. In human globulin, streptokinase causes the formation of an activator in excess, which produces high activities in bovine plasminogen. C. Similar high activities are produced in bovine plasminogen by the activator found in spontaneously active human blood.

Significant amounts of a specific streptokinase inhibitor could not be demonstrated in our preparations. Plasmin inhibitors cannot be the cause of the low activity observed in case *A*. Such inhibitors must be as effective in case *B* and *C* as in *A*. Hence the low activity in *A* must depend on an incomplete conversion of the bovine plasminogen to plasmin. This result excludes a direct reaction between streptokinase and plasminogen and presupposes the involvement of a plasminogen proactivator. The formation of a large excess of plasminogen activator in human globulin by streptokinase shows that a proactivator (as well as plasminogen) is present in human

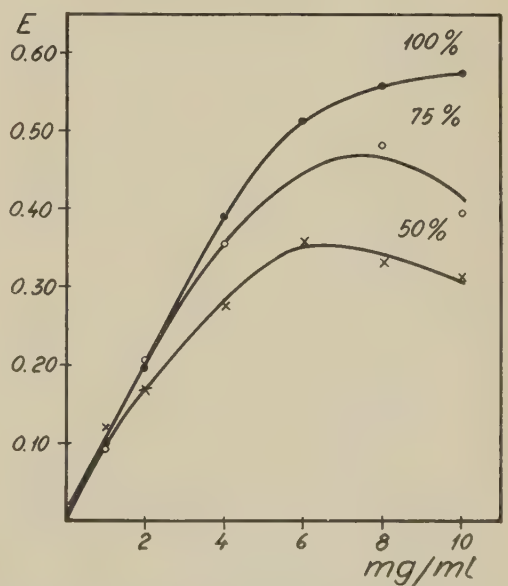
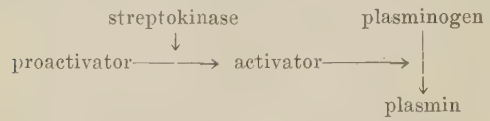


FIG. 4. Digestion of casein by increasing concentrations of plasminogen activated for 60 min. (37°C) by 3 different concentrations of streptokinase activated human globulin (dil. 1:200, streptokinase concentration: .012 mg/ml). Digestion time: 20 min. *Abscissa:* mg bovine plasminogen per ml. *Ordinate:* see Fig. 3. *Activity of controls.* Bovine plasminogen (10 mg/ml) + streptokinase (.012 mg/ml): .020. Streptokinase activated human globulin + buffer: .012.

blood. The formation of plasmin by streptokinase evidently follows this scheme:



Christensen(3) found the formation of plasmin in human globulin by the action of streptokinase to be a catalytic process. Therefore, at least one of the reactions mentioned here must be catalytic. The fact that preparations of bovine plasminogen are incompletely activated by streptokinase strongly indicates a stoicheiometrical reaction between the activator formed by streptokinase and plasminogen. Further evidence in support of this view was presented here (Fig. 4). Similarly, Astrup (11) found that plasminogen was activated stoicheiometrically by fibrinokinase.

Our experimental results are now easily understood. A complete activation of plasminogen by streptokinase can be reached only when sufficient amounts of plasminogen proactivator are present. Insufficient amounts are present in bovine preparations, especially in our bovine plasminogen preparation. Somewhat larger but still insufficient amounts are present in the fresh bovine globulin. Large amounts of proactivator are present in human globulin. Therefore, a large excess of activator is formed in human globulin by addition of streptokinase, and all plasminogen present is activated.

Bovine fibrin normally contains large amounts of plasminogen. In this substrate, therefore, activators contained in the test samples activate plasminogen of the substrate and produce activities, which are much larger than those corresponding to the plasmin content of the samples. Human fibrinogen contains a large excess of proactivator in addition to plasminogen. Therefore, the results of estimations of plasminogen or plasmin depend on the substrates applied and on the mode of activation of the samples. Discrepancies in previous streptokinase studies are caused by these facts. With bovine fibrin as substrate, preparations of human plasminogen activated by streptokinase produced activities much larger than those obtained with preparations from a number of other animals(4). With casein as substrate smaller differences only were found(12). With bovine fibrin as substrate, fibrinokinase produced activities of the same magnitude in human and bovine plasminogen(4). With the same substrate, human plasminogen was activated by streptokinase to activities several hundred times larger than those obtained with fibrinokinase(6). The observations presented here necessitate a reconsideration of all previous studies on the action of streptokinase.

The plasminogen activator recently demonstrated in spontaneously active blood obtained from the human organism was assumed to be formed from a precursor in blood(7). This assumption is also strongly supported by the present findings.

Summary. 1. Streptokinase transforms a proactivator in blood to an activator, which converts plasminogen to plasmin. Streptokinase does not react directly with plasminogen. 2. A low content of plasminogen proactivator in bovine blood is the reason for the incomplete activation of preparations of bovine plasminogen by streptokinase. In human globulin a large excess of plasminogen activator is formed by streptokinase. 3. The large activities usually observed by the activation of human globulin with streptokinase is caused by the action of this activator on the plasminogen contained in the fibrin substrate.

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Influence of Hyper and Hypothyroidism on Susceptibility of Mice to Infection with Lansing Poliomyelitis Virus.* (20088)

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In seeking an explanation for the seasonal variation in incidence of poliomyelitis and for the predilection of the disease for the lower age groups certain investigators have questioned the role of the thyroid gland. Clinicians have reported that chilling seems to be a predisposing factor in some cases of poliomyelitis. The experimental test of this clinical observation was provided by Milzer *et al.*(1). These workers found that rhesus monkeys inoculated intracerebrally with Stimpert's BK strain of poliomyelitis virus and then subjected to chilling in cold water exhibited a higher incidence and greater severity of paralysis than did the controls. Holtman(2) studied the effect of environmental temperature on susceptibility of mice to poliomyelitis virus. He reported that the higher the acclimation temperature before inoculation, the shorter was the incubation period of the virus. The incubation period was lengthened somewhat when the mice were maintained following inoculation at temperatures lower than those of acclimation. Since these results appear to conflict with those of Milzer *et al.*, Holtman stated that the difference might be due to the suddenness of temperature change. Manifestly, also, the disease in mice differs from that in the monkey. Since it is well known that thyroxine secretion is increased upon exposure to cold, Holtman tested the effects of thiouracil and thyroactive substances upon susceptibility of mice to poliomyelitis(3). Mice receiving thiouracil invariably showed signs of paralysis and died earlier than the controls. Administration of thyroid extract or thyroprotein produced incubation periods longer than those in the controls or the hypothyroid mice. Gollan(4) could demonstrate no differences in susceptibility of mice injected with thyroxine to MM virus. The experiments reported here were carried out in an attempt to clarify these controversial findings and to determine the influence of hyper- and hypothyroidism upon susceptibility of mice to the Lansing strain of poliomyelitis virus.

Materials and methods. Mice: Webster Swiss mice from our own colony were used in all experiments. Handling and care of the animals have been described previously(5). The average age of the mice in series 145 and 148 at the time of inoculation was 32.4 days, and in series 146 was 37.6 days. Diets: The basal or control ration referred to as "optimum" had the following percentage composition: vitamin-free casein 19, sucrose 71.2, salts IV 4, B vitamin mix 0.5, choline chloride 0.3, corn oil§ 5. The 0.5 g of B vitamin mix contained the following in sucrose: thiamine hydrochloride 0.3 mg, riboflavin 0.3 mg, pyridoxine hydrochloride 0.3 mg, nicotinic acid 1.5 mg, calcium pantothenate 2 mg,

TABLE I. Dietary Regimens.

Diet No.	Type and composition of diet
1	Optimum (control)
2	Hyper*—Opt + .25% IC 16 days, then .1% IC
3	Hyper —Opt + .1% IC 14 days, then .05% IC
4	Hyper —Opt + .06% IC
5	Hyper —Opt + .08% IC
6	Hypo —Opt + .1% PTU 7 days, then .02% PTU
7	"Hyper-Hypo"—Opt + .1% IC and .1% PTU 7 days, then PTU .02%

* Hyper = Hyperthyroid; Hypo = Hypothyroid; Opt = Optimum.

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§ Mazola, fortified with oleum percomorphum (Mead Johnson and Co., Evansville, Ind.) to supply *ca.* 1800 I.U. vit. A and 260 I.U. vit. D per 100 g ration.

TABLE II. Summary of Fatalities and Virus Infection.

Series No.	Virus dilution	Group No.*	Ration No.	No. of mice	Fatalities,† %	Virus infection,‡ %	Total fatalities, %	AIP,§ days	AST,‖ days
145	10^{-2}	1U	1	7	0	—	0	—	—
		1I	1	28	11	89	100	7.6	8.3
		2U	2	7	29	—	29	—	—
		2I	2	27	48	52	100	7.2	6.8
		3U	6	6	0	—	0	—	—
		3I	6	28	0	96	96	5.9	7.4
		4U	7	7	0	—	0	—	—
		4I	7	25	52	48	100	6.3	6.9
146	10^{-2}	1U	1	7	0	—	0	—	—
		1I	1	40	8	88	96	7.8	9.6
		2U	3	7	29	—	29	—	—
		2I	3	29	34	62	96	7.9	8.0
		3U	6	7	0	—	0	—	—
		3I	6	40	10	88	98	7.8	9.6
148	10^{-2}	1U	1	7	0	—	0	—	—
		1I	1	34	3	85	88	6.8	7.9
		2U	6	7	0	—	0	—	—
		2I	6	33	9	88	97	7.3	8.7
		3U	4	7	14	—	14	—	—
		3I	4	20	50	50	100	5.7	7.1
		4U	5	7	43	—	43	—	—
		4I	5	21	38	57	95	7.8	8.3
148	10^{-3}	1U	1	7	0	—	0	—	—
		1I	1	14	14	64	78	8.3	9.6
		2U	6	7	0	—	0	—	—
		2I	6	14	14	57	71	9.1	9.1

* U = uninoculated controls; I = inoculated. † Without signs of infection.
‡ Showed paralysis before death. § Avg incubation period in mice showing paralysis.
‖ Avg survival time of mice dying with or without signs of paralysis.

i-inositol 100 mg, sodium p-aminobenzoate 100 mg, biotin 10 micrograms, folic acid 25 micrograms. To provide bulk and reduce the hygroscopicity of the ration 10 g of Cellu flour were added per 100 g of diet. Iodinated casein (IC)‖ and 6-n-propyl-2-thiouracil (PTU)¶ were used to produce the hypothyroid and hypothyroid states, respectively. These substances were added to the diet at the expense of sucrose in amounts established by Miller and Baumann(6) for the rat. The compositions of the various diets are given in Table I. The "hyper-hypo" diet (7) contained both IC and PTU in a combination which produces only a slightly elevated BMR in the rat. In the hyperthyroid diets containing IC the amounts of thiamine, riboflavin, pyridoxine, biotin and folic acid were doubled and 7 μg vitamin B_{12} per 100 g of ration were included. Rations and water were fed *ad libitum*. *Virus.* In series 145 and 148 the mice received their respective rations for 7 days before inoculation and in series 146 for 11 days. In series 145 and 146 the inoculum was 0.03 ml of a 10^{-2} brain-cord suspension of Lansing virus, containing 100 LD50, administered intracerebrally. In series 148 dilutions of 10^{-2} and 10^{-3} were employed. All animals were observed daily, and the controls were weighed twice weekly.

Results and discussion. In Series 145 all of the hypothyroid mice (Group 3I, Table II) showed paralysis before death. The disease also appeared to be more fulminating in the hypothyroid animals than in the controls since incidence of paralysis and death of the former group exceeded that of the latter during the first 6 days following inoculation. The average incubation period (AIP) in the hypothyroid mice was somewhat less than in the controls. In the hyperthyroid group (2I) there was a high incidence of deaths without

‖ Protamone; Cerophyl Laboratories, Kansas City, Mo.

¶ Furnished by Calco Chemical Division, American Cyanamid Co., Bound Brook, N. J.

previous signs of paralysis. Many of these deaths were undoubtedly due to thyrotoxicosis since 2 of the 7 uninoculated control animals died. However, it is of interest that group 4I, which received IC and PTU in a ratio which maintains the BMR of rats only slightly above normal, manifested almost identical incidences of paralysis and fatalities without signs of paralysis as did the hyperthyroid group. It is also noteworthy that none of the uninoculated controls for this group died. The differences in AIP and AST between the various groups were not great, although it is evident that these figures for the normal group were greater than for any of the other groups, and that the shortest AIP was in the hypothyroid group. In series 146 the amount of IC in the diet of the hyperthyroid mice was reduced, but still 2 of the 7 uninoculated controls died. Again there was a high incidence of deaths without signs of paralysis in the inoculated group (2I). In this series the hypothyroid group (3I) did not differ from the normal group (1I).

The mice selected for series 148 were of the same age as those used in series 145, and a further virus dilution of 1:1000 was employed with normal and hypothyroid animals. There were no differences between normal (1I) and hypothyroid (2I) mice at either virus dilution with the exception of a slightly prolonged AIP in the latter. In both of the hyperthyroid groups (3I and 4I), which received less IC than was fed in the other 2 series, the incidence of fatalities without signs of paralysis was high. In group 3U only 1 of the 7 controls died, whereas in the inoculated group (3I) 50% died without signs of paralysis. This seems to bear out the results obtained in series 145, namely that even a slightly hyperthyroid condition in mice either favors the proliferation of virus or imposes sufficient stress on the already taxed metabolism to cause death in a large percentage of the animals before signs of paralysis are evident.

The results represented here are at variance with those obtained by Holtman, although in series 145 the hypothyroid condition did seem to favor slightly the progress of the disease, further experiments failed to substantiate this. Furthermore, the administration of thyroprotein did not increase the incubation period, but it did bring about a high incidence of deaths without signs of paralysis. It may well be that the virus employed by Holtman was not affected in the same way by the BMR of the host as is the Lansing strain of poliomyelitis. It is also possible that as Holtman has suggested there is a narrow critical range within which variation in the metabolic rate may alter susceptibility to poliomyelitis. More precise experiments should determine if such a critical range actually exists.

Summary. The influence of hyper- and hypothyroidism upon susceptibility of mice to infection with Lansing poliomyelitis virus has been studied. In one series hypothyroidism seemed to favor a higher incidence of paralysis, but this was not substantiated in two other series. The only consistent deviation from the normal course of infection was an increased incidence of deaths without previous signs of paralysis in hyperthyroid mice.

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Significance of the Neurohypophysis in Regulation of Fluid Balance in the Frog.* (20089)

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It has been known since 1921(1) that neurohypophyseal extracts cause a temporary weight increase in frogs. It is now well established that this "water-balance effect" is due to an increased rate of water uptake through the skin(2). Heller(3) states, however, that there is insufficient evidence on which to base a theory as to the physiological significance of the water-balance effect. In this paper it is demonstrated that the frog neurohypophysis releases its hormone in response to dehydration, and that this hormone can increase the rate at which dehydrated frogs regain water loss. These facts are presented as evidence for the physiological importance of the neurohypophysis in the regulation of the water economy of the frog.

Methods. Male *Rana pipiens* averaging about 30 g were used. The technics of handling, weighing, hypophysectomy, and statistical treatment have been described previously (4). Contraction of the melanophores of the skin with subsequent inability to expand in response to dark adaptation was used as the criterion of successful total hypophysectomy. The frogs were dehydrated by placing them in individual, dry, wire-covered beakers in a tightly closed closet. To measure rehydration, 100 ml of water was added to each beaker. Neurohypophyses were removed with a generous amount of adjacent nervous tissue and kept frozen until assayed. Individual glands were homogenized and extracted by boiling in 1 ml 0.25% acetic acid for 5 minutes, centrifuged, and the supernatant used for assay. Oxytocic activity was determined by a modified Holton rat uterus method(5). The accuracy of this method is approximately ±10%.

Results. A. *Response of neurohypophysis to dehydration.* We have attempted to determine whether the neurohypophyseal hormone is released in response to the need for water conservation. To this end we have assayed the hormone content of the hypophyses of dehydrated and control frogs. Direct estimation of the water-balance activity requires a large number of frogs and is not practical for the assay of individual frog pituitaries. Since the frog water-balance activity resides in the oxytocic fraction of mammalian neurohypophyseal extracts(6) we have used an oxytocic assay. Frogs were rapidly dehydrated under an electric fan until they had lost about 25% of their original body weight. They were then maintained at this weight for 48 hours in a saturated atmosphere over water. Control frogs were kept in water throughout the experiment.

TABLE I. Effect of Dehydration on Hormone Content of Neurohypophysis.

	Hormone in mu/gland	No. of frogs	Dehydration in % wt lost
Control	40 ± 7.3	13	—
Dehydrated	9.7 ± 2.0	12	26.3

It can be seen that the activity of the pituitaries of dehydrated frogs is only about one-fourth as great as that of control glands (Table I). In spite of the great variability a significant difference exists ($P < 0.01$). It is clear, therefore, that the frog neurohypophysis releases its hormone in response to the need for water conservation.

B. *Function of the neurohypophysis in dehydration.* In order to determine the possible physiologic role of the neurohypophysis in states of water deprivation we have compared the rates of dehydration and rehydration in hypophysectomized and intact frogs. The experiments on hypophysectomized frogs were always carried out the day following operation. Thus interference by the decrease in water-balance response, which appears only several days after hypophysectomy(4), could be avoided. Frogs were dehydrated for 24 hours, and then returned to water. Rates

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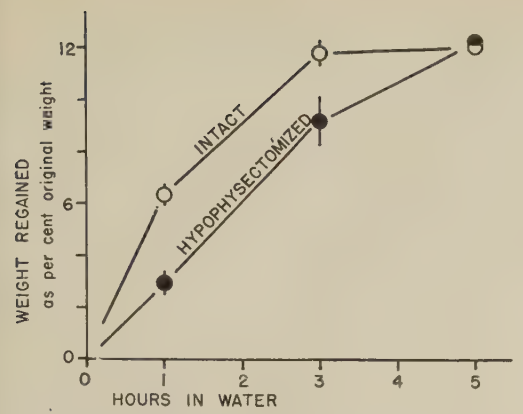

FIG. 1. Return to initial wt after dehydration of normal and hypophysectomized frogs placed in water. Means are represented by points, and stand. errors of the means by vertical lines through the points.

of dehydration and rehydration were determined by noting weight changes every two hours. The results represent pooled averages from three identical experiments, using a total of 31 hypophysectomized and 36 control frogs.

There is no significant difference in the rates at which hypophysectomized and control frogs lose weight during dehydration. Hypophysectomized frogs are not more prone to dehydration than control frogs. However, the rate of rehydration of intact frogs is considerably more rapid than that of the hypophysectomized frogs (Fig. 1). The differences in weight regained at one and at three hours are statistically significant ($P < 0.01$). Normal frogs regain the lost water within three hours while hypophysectomized frogs do not regain full weight until five hours after the return to water. It therefore seems clear that, although the neurohypophysis does not alter the rate of dehydration of the frog, its presence speeds the rate of rehydration when water becomes available.

Discussion. The function of the neurohypophysis in the regulation of fluid balance in mammals has been clearly defined experimentally. A similar function for the presumably homologous amphibian neurohypophyseal hormone has not been demonstrated as clearly. Thus, for example, Simon(7) showed that the pituitaries of dehydrated rats contain significantly less pressor and oxytocic activity than those of control rats. This has been interpreted as signifying that the neurohypophysis of the rat responds to the need for water conservation by releasing stored hormone. In the case of the frog Heller(3) stated that "no significant difference between the water-balance activity of glands of normal and dehydrated frogs" could be demonstrated in a small series assayed. Such results would seem to suggest that the hormone of the frog neurohypophysis is not functionally homologous to the mammalian antidiuretic hormone with respect to water conservation by the organism. The results presented in this paper, however, indicate that a definite difference in the hormone content of the glands of normal and dehydrated frogs does exist. In view of the large variability in our series it is understandable that the differences might not be apparent in a smaller series, particularly when assayed by the water-balance response of frogs, which in itself is highly variable. In any case, our results seem to establish that the frog neurohypophysis, like that of the mammal, releases its hormone in response to the need for water conservation. This is in harmony with Hild's(8) observations on the depletion of neurosecretory material, presumably representing the hormone, from the neurohypophyses of dehydrated frogs.

Granting that water-balance hormone is released in the frog in situations of water deprivation, the physiological utility of the hormone to the organism has been questioned. It has been shown that large doses of pituitrin can decrease the weight loss of frogs kept in a dry environment(9). Heller, however, has found, and we have confirmed, that hypophysectomized frogs are not more prone to dehydration than normal frogs. From these observations it would appear that the amount of hormone released physiologically is not sufficient to help the organism conserve water. From the data presented it does appear, however, that physiologically released hormone can substantially increase the rate at which dehydrated frogs can regain lost body water. We have previously demonstrated that relatively small doses (1.0 unit/100 g) of pitocin can increase the rate of entry of water through frog skin(6). This effect

probably accounts for the observation that normal frogs can regain lost body water faster than hypophysectomized frogs. In any case, this observation suggests strongly that the release of hormone from the neurohypophysis can serve a physiologically useful purpose in the frog. In the toad, which is much more sensitive to the antidiuretic effect of pituitrin(10), it would appear likely that the neurohypophysis could exert a significant effect on the rate of water loss as well.

Summary. 1. The hormone content of the frog neurohypophysis is depleted by dehydration of the animal. 2. Normal and hypophysectomized frogs become dehydrated at the same rate. Normal frogs, however, regain the body water lost by dehydration more rapidly than do hypophysectomized frogs. 3. These observations suggest that the neurohypophysis plays a physiologically significant role in the regulation of the water balance of the frog.

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In vitro Effects of Cortisone on Multiplication of Influenza B Virus.* (20090)

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Infections with a wide variety of organisms including protozoa(1), fungi(2), bacteria (3,4), and viruses(5,6) may be increased in severity by the administration of cortisone to the host. This virtually universal effect remains unexplained despite evidence that cortisone suppresses such physiological host reactions as inflammation(7) and antibody formation(8), depresses granulocytosis(9), and induces lymphocytolysis and the dissolution of lymphoid tissue(10). Although many studies of cortisone and infection have been concerned only with host mortality and disease, some have demonstrated increased concentrations of the infecting agent(3,4,11). It appeared of obvious importance to establish whether an increase in an infecting agent might be induced by cortisone in host tissue isolated from the influence of collateral cortisone effects on phagocytic infiltration and antibody reaction. Accordingly, the present study was undertaken of the multiplication of Influenza B virus in isolated sections of chick chorioallantoic membrane.

Materials and methods. Virus. Influenza B virus (Lee strain) was used. Aliquots of the same seed pot, stored in glass at —65°C were used throughout the study. 0.1 ml of $10^{7.9}$ or $10^{6.9}$ E.I.D.$_{50}$ of virus solution (undiluted and 10-fold diluted allantoic fluid) was inoculated into 2 ml of the following medium to give initial virus concentrations in the nutrient fluid of $10^{6.6}$ and $10^{5.6}$ E.I.D.$_{50}$ per ml. *Culture medium.* Equal volumes of modified glucosol solution as described by Fulton and Armitage(12) and phosphate buffer adjusted to 7.3 pH were mixed immediately before use and penicillin and streptomycin were added to yield final concentrations of 10 units and 40 mg/ml as described by Tamm *et al.*(13). This solution was distributed in test tubes (18 × 150 mm) in amounts of 2 ml. Tubes were stoppered to prevent evaporation. *Tissue*

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TABLE I. Distribution of Log_2 of Titer Ratios with t Values and Mean Titers of Cortisone and Control Groups.

Initial EID_{50}	Incubation, hr	Log_2 T ratios* −2	−1	0	1	2	3	Total N	Student's t, ts† vs to‡	Mean titer§ Cortisone	Control
$10^{6.6}$	0									24	24
	12		2	10	2	1		15	.280 < 2.977	35	28
	24		3	7	5			15	.693 < 2.977	55	51
	36		1	8	10	1		20	3.58 > 2.861	71	49
	48			6	9	4	1	20	5.10 > 2.861	83	41
	60			3	11	5	1	20	7.01 > 2.861	76	37
$10^{5.6}$	0									2	2
	12	2	2	9	2			15	.354 < 2.977	17	12
	24			11	4			15	2.25 < 2.977	49	42
	36		1	7	9	3		20	3.90 > 2.861	68	48
	48			3	10	2		15	6.05 > 2.977	62	32
	60			1	10	3	1	15	6.84 > 2.977	52	28

* T ratio: Titer ratio of cortisone and control tubes.
† ts: Sample value of t when $M = 0$.
‡ to: $Pr\{|t| > 2.977\} = .01$ when degree of freedom is 14.
$Pr\{|t| > 2.861\} = .01$ " " " " " 19.
§ Reciprocal of virus, arithmetic dilutions.

specimens. Specimens of chorioallantoic membrane measuring approximately 13 × 13 mm were taken from White Leghorn eggs of 10-12 days incubation. One membrane sample was added to each tube, and 5 tubes containing specimens from as many different eggs were used in each group in each experiment. *Cortisone.* (Free alcohol; 11-dehydro-17-hydroxycorticosterone, Merck†). A saturated aqueous solution of 280 μg/ml was prepared and sterilized by heating at 100°C for 30 minutes. 0.1 ml of this solution was added to the culture medium to give a final concentration of 14 μg/ml of nutrient fluid. An equivalent volume of distilled water was added to control test tubes. *Virus titrations.* Virus concentrations were measured by the conventional hemagglutination technic, employing serial 2-fold dilutions of 0.2 ml volumes of culture media and equal volumes of 1% human "O" RBC. Tubes were usually read independently by both investigators after a one hour period at room temperature. *Incubation of cultures.* Groups of test and control tubes inoculated with virus were incubated in a Warburg water bath adjusted to 35°C and were shaken at a rate varying from 70 to 95 strokes/minute with a stroke amplitude of 5 cm. After incubation for various time periods, as described below, 0.2 ml of the medium was withdrawn for hemagglutination titration and the same amount of fresh medium added to maintain a constant volume.

Design of experiments. Early experiments confirmed the finding of Tamm(14) that great variation in virus production might occur among membrane specimens from different eggs; therefore, care was taken to distribute tissues from each egg used through all experimental groups, and statistical analysis of experimental results has compared only pairs (cortisone and control tubes) containing membranes from the same egg. As 2-fold dilution series have been used in viral titrations, titer ratios of comparable pairs have been expressed as the logarithms (to the base 2) of the arithmetic dilutions in order to expedite analysis of data.

Experimental results. Control studies. To determine experimental error, 2 series of tubes containing the same amounts of virus (and no cortisone) were incubated and virus titrations performed at 45, 60, and 72 hours, and the distribution of the log_2 titer ratios of comparable pairs was examined. Statistical analysis revealed (with less than 1% risk) that both groups ($10^{6.6}$ and $10^{5.6}$ initial $E.I.D._{50}$) belonged to the same population, the mean of which is zero. Therefore, the 2 groups were combined and discardable values calculated

† Cortisone was generously supplied by Merck and Co., Rahway, N. J.

(with 1% risk). Values lay outside the following range: $1.54 \geqq \text{Log}_2$ T ratio $\geqq -1.40$. Thus, under the same conditions, or if no variable (*e.g.* cortisone) is added in one series as in this experiment, the Log_2 titer ratios will not be expected to exceed ± 2.

Effect of cortisone. Comparative viral increases with time in control and cortisone-containing tubes are summarized in Table I, in which mean hemagglutination titers derived from 15-20 separate titrations in 3-4 experiments are tabulated. It is evident that no essential differences in viral concentration are seen until the 36th hour of incubation, at which time and thereafter significant differences in virus titer are observed between control and cortisone-treated groups. Evidence for the statistical significance of the titer differences at 36, 48, and 60 hours is presented in Table I, in which the distribution of the Log_2 of the titer ratios of cortisone to control tubes is illustrated. It is notable that only twice in 110 titrations in the later time periods was the titer ratio less than ½ (indicating the possibility of less virus in the cortisone tube), and in most instances the concentration of virus was 2-fold or more greater in cortisone than in control tubes. Experiments using an initial inoculum of $10^{4.6}$ $E.I.D._{.50}$ have demonstrated results comparable to those described above with larger viral inocula. Preliminary experiments with 10-fold greater concentrations of cortisone have demonstrated slightly less viral increase than observed with the smaller amount.

Egg infectivity titrations. Despite marked differences in the amounts of virus as measured by hemagglutination, egg-infective virus decreased in amount in both cortisone and control groups after 60 hours of incubation. $E.I.D._{.50}$ were $10^{3.5}$ and $10^{3.0}$ at the end of one experiment, although the initial $E.I.D._{.50}$ had been $10^{6.6}$ and $10^{5.6}$. There were no significant differences in the infectivity titers of cortisone and control groups, as might be expected with this less precise method.

Histologic study. After 60 hours of incubation, chorioallantoic membranes were fixed in Zenker's solution and stained with H and E. Microscopic examination revealed no differences between cortisone-treated and control membranes.

Bacterial contamination. In the course of 9 experiments including 198 tubes no instances of bacterial contamination have been revealed by aerobic culture on blood agar plates of culture fluids following the completion of experiments.

Direct effect of cortisone on viral hemagglutinin. Incubation of virus and cortisone in the absence of tissue has shown no evidence of any effect of cortisone on the concentration of hemagglutinating virus.

Discussion. It has been demonstrated that the addition of small quantities of cortisone to a tissue culture medium results in significantly greater final concentrations of Influenza B virus than observed in cultures without cortisone. Studies of virus concentration at varying time intervals have not revealed differences in the *rate* of increase; however, but instead have indicated continued increase of virus in cortisone cultures in the later time periods. Experiments in this laboratory have shown that cortisone in appropriate dosage may increase the survival time of Lee virus-infected chick embryos(15) and decrease the pathological reaction in the chorioallantoic membrane(16) despite a concomitant increase in virus greater than observed in shorter-surviving control eggs. It is suggested, therefore, that the increase in viral concentration induced by cortisone in the present study may result from prolonged survival of membrane cells permitting continued viral multiplication after "death" of control tissue has occurred. Histologic study of membranes at the crucial 36-hour period may adduce further evidence for this hypothesis.

Summary. The addition of cortisone to chick embryo chorioallantoic membrane tissue cultures results in significantly greater final concentrations of Influenza B virus than observed in controls.

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Further Studies on Oral Administration of Living Poliomyelitis Virus to Human Subjects. (20091)

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In a previous study(1) the results of oral administration of living poliomyelitis virus to 20 human volunteers were reported. In most of the subjects the virus was recovered from the stool; 16 subjects who were not immune prior to the feeding developed Lansing type neutralizing antibodies in their blood; none of the subjects showed any apparent untoward effects from the administration of the virus nor was any febrile response noted.

In the present communication, the results are reported of the effects of oral administration of the same type of virus to 61 human subjects, all of whom had no Lansing antibodies prior to the experiment. It will be seen that the results obtained in the first trial have been confirmed and to a certain extent amplified.

Materials and methods. The subjects for experiment were selected in the following way: 181 mentally defective children ranging in age from 8 months to 8 years were bled in April, 1952, and the sera tested for neutralizing antibody content against the MEFl strain, Type II poliomyelitis virus (see below). The children were inmates of a State institution for mental defectives. Of the children who had no antibodies, 61 were selected for the trial after permission of each child's parents had been legally obtained. Each child was tested again for presence of neutralizing antibodies during the last week of June, 1952, and fed the virus on July 1. During the following period of 21 days the children were placed in an isolation unit under constant medical observation and nursing care. Rectal temperatures were taken every 8 hours and search was made daily for development of somatic disturbances with particular attention to the central nervous system. Stools for virus isolation were collected from every treated child on the 5th, 8th, 12th, 15th, 18th and 21st days after feeding. Blood for virus isolation was obtained from each subject by venous puncture. The patients were divided into two groups and members of each group were bled on alternate days for 12 days, starting on the first day after feeding. Thirty days following virus feeding, all patients were bled and the sera submitted to

* In cooperation with the California State Departments of Mental Hygiene and Public Health, and the George Williams Hooper Foundation, University of California, San Francisco.

neutralization test. *Virus strains.* The TN strain(1,2) of rodent-adapted poliomyelitis virus was used for feeding purposes. It was administered in the form of a 20% suspension of infected cotton rat brain and spinal cord tissues prepared exactly as described elsewhere(1). Two pools of infectious material were used: One (No. 24) represented the 35th cotton rat passage, and the other (No. 31) represented the 2nd cotton rat passage of the virus(1). Each feeding dose consisted of 5 ml of the suspension diluted with 2 oz of chocolate milk for children, or feeding formula for infants. The MEFl strain of poliomyelitis virus(3) was used for the neutralization test.

Methods for examination of stool and blood. Stool specimens were processed according to the method described before(1) and were assayed for the presence of virus by intracerebral inoculation of 10 Swiss albino mice per specimen. If signs of paralysis were noted in any of the inoculated animals, the affected mice were sacrificed and the brain and spinal cord tissues subinoculated into another group of 8 mice. In every case the identity of the agent isolated by the mouse passage technique was confirmed by neutralization test against Lansing type-specific hyperimmune serum obtained from a rhesus monkey recovered from paralytic infection(1). Blood specimens were injected intracerebrally into mice within 4 hours after withdrawal. Each sample was injected into 10 mice. When none of the inoculated animals showed signs of illness, one or two animals were sacrificed on the 7th and 14th day after inoculation and their brain and spinal cord tissues subinoculated into other groups of mice. Following this, another "blind" passage was made and the virus was considered to be absent in the patient's blood if neither the inoculated nor the subinoculated groups of mice showed signs of illness. *Neutralization test.* The technic of the test followed that described previously (1), except that in most cases only one dilution of virus was used against serial dilutions of serum.

Results. Distribution of antibodies against Type II poliomyelitis virus. Out of 181 patients bled in April, 1952, undiluted sera of 106 failed to neutralize the MEFl strain of poliomyelitis virus. Sera of 10 did neutralize the virus if tested in undiluted form but were devoid of neutralizing power when diluted 1:3; sera of 21 gave partial protection in undiluted form and in 1:3 dilution. Thus, 31 patients were classified in the "doubtful" category. Sera from the remaining 44 patients neutralized the virus when tested both in undiluted form and in 1:3 dilution. Of the 106 inmates whose sera showed no neutralizing power against Type II virus, 61 were ultimately selected for the trial and were fed the virus.

Lack of clinical manifestations of poliomyelitic infection. None of the 61 children showed any apparent signs or symptoms of illness which could be attributed to the ingestion of the virus. A few children developed elevated temperature which lasted only a few hours. The cause was promptly recognized in some trivial condition such as constipation, mild tonsillitis, nasopharyngitis, etc., and prompt response was obtained with adequate therapy. In 3 cases, diarrhea developed and stool culture demonstrated the presence of a Shigella organism. In no patient was there any evidence of meningeal irritation or of paralytic changes.

Absence of viremia. No virus was isolated from any of the blood specimens obtained from the patients during the 12 days following oral administration of the virus.

Antibody response. The results are summarized in the accompanying table. It may be observed that a virus identified as TN strain was isolated from 53 stool specimens obtained from 29 of the 61 patients. In 16 of these patients, TN virus was isolated from only one stool specimen, in 6 patients from 2 stools, in 4 patients from 3 stools, in 2 patients from 4 stools and in 1 patient from 5 stools. It may also be observed that the antibody rise against Type II poliomyelitis virus was significant in most of the cases. No neutralizing antibodies were present in the sera of 6 patients (Nos. 8, 16, 43, 51, 54, 57) 30 days after feeding with virus, and only one of these patients (No. 8) excreted virus in his stool, *i.e.,* that which was collected on the 5th day of the trial. No sig-

TABLE I. Occurrence of Intestinal Carriage and Serological Evidence of Immunity in 61 Human Subjects after Oral Administration of TN Strain of Poliomyelitis Virus.

Virus isolated from stools (days postfeeding)	50% protective serum titer§ Prefeeding	Postfeeding	Virus isolated from stools (days postfeeding)	50% protective serum titer§ Prefeeding	Postfeeding
*8	0†	>1:600	15	0‡	1:126
5, 8, 15	1:4‡	1:200	18	0	1:294
*0	1:2‡	1:174	0	0	1:119
5, 8	1:10	1:398	20	0	1:165
15	0	1:210	12, 15	0	1:18
5	0	1:1030	5	0‡	1:105
*8	1:4‡	1:256	0	0‡	1:27
5	1:4‡	0	0	1:3	1:204
*5, 8	1:5‡	1:318	20	0	1:13
0	0	1:161	12, 15, 18, 20	1:3	1:105
12	0	1:822	0	1:2	1:24
0	0	1:344	0	0	0
0	0‡	1:80	8, 18	0‡	1:33
12, 18	0	1:250	0	0	1:39
0	0	1:20	0	0‡	1:127
0	0‡	0	8	0	1:18
18	0	1:31	0	1:4	1:127
0	0	1:22	18	0	1:328
0	0	1:340	0	0	1:468
0	1:4	1:131	0	0‡	0
*0	0‡	1:16	0	0	1:154
0	1:4	1:250	0	1:10‡	1:12
0	0	1:586	0	0‡	0
*0	1:27‡	1:22	5, 8, 12, 15, 18	0‡	1:362
5, 18	0‡	1:318	0	0	1:50
0	0	1:58	0	0‡	0
*5, 8, 15, 18	0‡	1:248	18	0	1:21
5, 8, 12	0‡	>1:250	0	1:26‡	1:71
0	0‡	1:145	12, 15, 18	0‡	1:48
*0	0‡	1:26	5, 8, 15	0	1:39
15	0	1:80			

* These individuals were fed with virus pool #24; all others received pool #31.
† 0 = <1:2 titer.
‡ Repetition of neutralization test gave similar results.
§ Based on the cumulation titer of the virus pool; 76 LD_{50} were used in neutralization test.

nificant rise in the antibody level was observed in the blood of 3 patients (Nos. 24, 53, 59) after administration of the virus, although the sera of these 3 patients seemed to show some neutralizing power when collected before ingestion of the virus. The antibody rise in the remaining 52 patients was significant enough to consider their immunological response adequate.

Comments. The results of the present study indicate that no clinical signs of illness were elicited by feeding the living TN strain of poliomyelitis virus to 61 children and thus confirm and amplify findings described in the previous paper(1) in which a similar lack of clinical manifestations was observed in 20 human volunteers fed the same virus. It is interesting to note that 30 of the present subjects fed the virus disclosed evidence of preexisting severe brain damage, a condition which in the past has been considered to be a predisposing factor to the paralytic form of poliomyelitis(4). Yet none of the patients developed new neurological manifestations following feeding. In this respect it is pertinent to observe that although epidemic form of poliomyelitis had been present in the immediate vicinity of the institution for many years, no cases of paralytic poliomyelitis had ever been noted among the institutionalized children(5). That this fact can be explained on the assumption of a high incidence of the subclinical form of the disease among the inmates is not supported by the finding of a high percentage (60%) of non-immune individuals among the 181 tested prior to the trial. It should be noted also that 19 children were affected by mongolism, a condition

which has been considered to hinder formation of antibodies(6). Yet the immune responses in mongolians were not significantly different from those of the other children.

The finding that viremia was not observed in any of the 61 subjects should be emphasized in view of the recently advanced theory of Bodian that the absence of virus in the circulating blood precludes the development of paralysis(7).

Although the presence of virus in the stool could be demonstrated in 29 out of 61 patients, the antibody response would indicate that infection was established in many more cases, since the sera of only 6 patients had no neutralizing antibodies on the 30th day after ingestion of virus. One may possibly assume that in 5 out of these 6 patients the virus was destroyed before reaching the intestinal tract since no virus was isolated from their stool specimens. There was either insignificant or no rise in antibody response in 3 other patients whose blood did show some neutralizing power when drawn prior to feeding with virus. However, if one may draw a theoretical parallel with results obtained in cynomolgus monkeys(8), the level of antibodies observed in the latter 3 patients should be considered high enough to prevent paralysis after a peripheral exposure to virulent Lansing type virus. In any event, perhaps another oral administration of the TN strain to the 9 patients would result in multiplication of the virus in the intestinal tract and subsequent adequate serological response. On the other hand, a study of the genetic background of these 9 patients may throw additional light on their "non-reactivity" to the infectious process. The recent accumulation of data on the relationship between susceptibility to the paralytic form of poliomyelitis and the genetic constitution of the individual (9-11) may warrant investigation of the familial background of the 9 patients.

Summary. Sixty-one children who had no antibodies against Type II (Lansing) poliomyelitis virus were fed the TN strain of virus. None showed any clinical signs of illness. Viremia was absent in every case. In 29 individuals the virus was isolated from the stool from the 5th to the 20th day after feeding. Specific antibody rise was observed in most of the patients.

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Heparin-Like Anticoagulants from Mollusca. (20092)

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In the present investigation a study of a wide variety of aquatic life forms such as carp, whitefish, scallops, clams, and oysters revealed that 2 species of clams, *Mactra spissula* (sea surf clam) and *Artica islandica* (ocean quahog clam), contain unusually large amounts of heparin-like substances which exhibit high anticoagulant potency and low

TABLE I. Yield of Anticoagulant Activity in Crude Clam Extracts.

Species	Amt extracted, kg	Wt of crude extr., g	Anticoagulant activity of crude extr., U.S.P. units/mg	Anticoagulant activity/kg of meat, U.S.P., units × 100
Mactra spissula	82	310	10	374
	84	59	38	264
	91	747	6	484
Artica islandica	39	25	31	198
	86	864	4	396
	91	470	7	352

toxicity. We have designated the anticoagulant sulfated polysaccharides derived from *Mactra spissula* and *Artica islandica* as "mactin-A" and "mactin-B," respectively, the term being derived from the generic name of the sea surf clam.

This report deals with the extraction, purification, and certain biological, chemical, and physical characteristics of mactin-A and mactin-B.

Experimental. Isolation and purification. Crude extracts were prepared from the autolyzed meats of clams by alkaline extraction and subsequent proteolytic digestion. Representative activities and yields of the crude extracts are presented in Table I. All *in vitro* activities were determined by the U.S.P. sodium heparin assay(1) using sheep plasma. For comparison, the reported average yield of heparin activity from beef lung, one of the richest mammalian sources, was 18700 U.S.P. units per kg(2). An effective method of purification consisted of the following steps: The crude material obtained by precipitation with ethyl alcohol after proteolysis was dissolved in dilute alkali, then acidified to pH 1.5; protein impurities were denatured with gentle heating and removed by filtration; active material was precipitated in the filtrate by addition of ethyl alcohol, separated by centrifugation, redissolved in water, and finally isolated as a sodium, calcium or barium salt by addition of acetic acid. Purified products have been obtained with *in vitro* anticoagulant activities which range from 70 to 120 U.S.P. heparin units/mg. Electrophoretic analyses (Fig. 1) of these purified samples demonstrated the presence of several components.

In vivo activity. The results of anticoagulant studies showed that both mactin-A and mactin-B possessed greater *in vivo* activity in New Zealand white rabbits than was demonstrated by the *in vitro* U.S.P. sodium heparin assay. The data in Fig. 2 represent average values obtained with mactin-B from 4 or more rabbits. Similar results were obtained by intravenous injection of equivalent doses in cats.

Toxicity studies. Purified samples of mactin-A and mactin-B with *in vitro* activities from 70 to 100 U.S.P. units/mg were used in all the following experiments. 1) The LD_{50}* in mice of a sodium salt of mactin-A was found to be 1.6 g/kg compared to 2.8 g/kg for standard U.S.P. sodium heparin. 2) *Acute toxicity.*† Single intravenous or subcutaneous injections of 10, 30, 50, and 100 mg/kg pro-

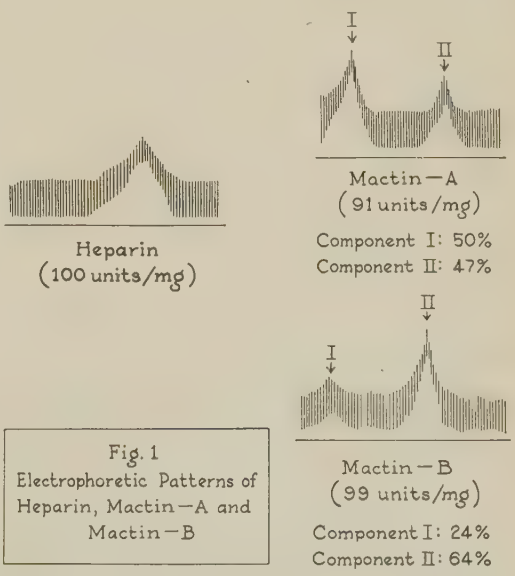

Fig. 1
Electrophoretic Patterns of Heparin, Mactin—A and Mactin—B

* Determined by Department of Pharmacology, Lederle Laboratories.

† Determined by Pharmaceutical and Chemical Testing Department, Lederle Laboratories.

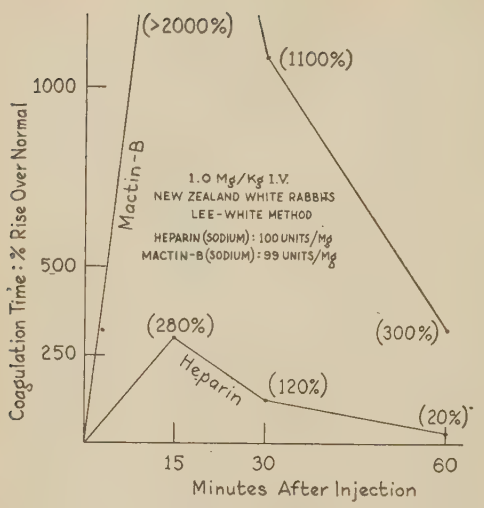

Fig.2 In-Vivo Activity of Mactin-B and Heparin in Rabbits

duced no toxic effects in 2 New Zealand white and 2 Dutch belted rabbits. Two monkeys injected intravenously on 3 successive days with 2, 5, and 10 mg/kg exhibited no toxic reactions. No toxicity was observed in 50 mice and 40 rats after 5 daily subcutaneous injections of 10 mg/kg. 3) *Chronic toxicity.* Eight rats were injected intravenously with 20 mg/kg twice daily for 6 weeks with no evidence of hemorrhage or adverse effects on weight, activity, and appetite.

4) *Anaphylaxis.*† Three groups of 10 guinea pigs each were injected intraperitoneally with a sensitizing dose of 10 mg/kg. A challenging dose of 10 mg/kg administered 10 days later produced no anaphylactic symptoms. No antigenic effects have been noticed in any of the animals tested.

5) *Blood pressure.*† The blood pressure of 2 cats (histamine test) was not affected by intravenous doses of 5 and 10 mg/kg. 6) *Platelet count and agglutination.* A slight tendency of platelets to agglutinate in several clusters of 3 to 5 cells was first noted in New Zealand white rabbits at 10 mg/kg 15 minutes after injection; however, no decrease in platelet count was evident and agglutination was absent 30 minutes after injection. Neither platelet decrease nor agglutination occurs in the therapeutic range with highly purified samples. Further investigations of the effect on platelets of mactin-A and mactin-B at higher levels and under sustained conditions are in progress. 7) *Sedimentation.* No change of sedimentation rate has been noted in the blood of any of the animals tested.

Discussion. The crude product obtained after alkaline extraction and proteolytic digestion of both species of clams contained unexpectedly large quantities of anticoagulant activity determined by *in vitro* assay. In general, the yield of activity in crude clam extracts was found to be approximately twice the heparin activity obtained from a comparable weight of beef lung.

Since the electrophoretic patterns demonstrated the presence of at least 2 components in the purified samples of both mactin-A and mactin-B, it is obvious that the results of investigations concerning composition and structure may be subject to revision. However, a number of the tests used to determine the nature of heparin were applied to mactin-A and mactin-B. As with all sulfate esters of polysaccharides, a strong metachromatic color shift was obtained with both toluidine blue and azure-A. Carbohydrate color tests with mactin-A or mactin-B gave results similar to those obtained with heparin. Elemental chemical analyses have demonstrated a somewhat lower sulfur content for mactin-A and mactin-B than for heparin. Comparison of infra-red spectra of heparin, mactin-A, and mactin-B showed that the molecules were similar, but differences were noted in the positions of the absorption bands at wavelengths above 8 μ. The optical rotations of the sodium salts were almost identical with that of heparin. However, the intrinsic viscosities of both mactin-A and mactin-B were greater than that of heparin, which might be interpreted as evidence of higher molecular weight. In an effort to gain more information concerning the relative molecular weights of heparin and mactin-A and mactin-B, the technics of light scattering and the ultracentrifuge are being investigated.

Although a crystalline acid barium salt may readily be prepared from 100-unit heparin, all attempts to crystallize a corresponding acid barium salt of mactin-A and mactin-B with *in vitro* anticoagulant activities of approximately 100 U.S.P. units/mg have been un-

successful.

In vivo results in rabbits and cats revealed that sodium salt preparations of mactin-A and mactin-B with *in vitro* activities of approximately 100 units/mg were more active than preparations of standard 100-unit sodium heparinate. The observed intensity of action was more than 6 times that of heparin; the duration of action was also significantly greater. A satisfactory explanation for this wide discrepancy between the *in vitro* and *in vivo* activities has not been found.

As in the case of heparin the anticoagulant effect of mactin-A is neutralized by injection of protamine.

While the LD_{50} of mactin-A of 1.6 g/kg is lower than the value of 2.8 g/kg established for heparin, the anticoagulant activity of mactin-A is greater than that of heparin; therefore, the therapeutic index of mactin-A would appear to be comparable to that of heparin. Acute and chronic toxicity studies conducted thus far have revealed no apparent toxicity. Clinical trials of mactin-A and mactin-B are in progress.

Summary. Heparin-like polysaccharides with high anticoagulant activity have been isolated from 2 species of clams, *Mactra spissula* and *Artica islandica*. These purified polysaccharides, termed mactin-A and mactin-B, are related to but not identical with mammalian heparin. They possess a greater *in vivo* activity and a favorable therapeutic index when compared with heparin preparations of equal *in vitro* activity.

We wish to thank Dr. Raymond Brown for the electrophoretic analyses, and Mr. L. Brancone and associates for microchemical analyses.

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Liver Regeneration: Preliminary Report.* (20093)

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The reason for the failure of hepatic regeneration following partial hepatectomy in dogs with Eck fistulae is not clear. Some relate this to diversion of a factor peculiar to portal blood and others simply to reduction in total blood flow through the liver. In an attempt to discover whether portal blood is essential to hepatic regeneration an operation was devised in dogs that accomplished complete diversion of the portal stream and at the same time provided the liver with a profuse supply of systemic venous blood. In this preparation the response of the liver to partial hepatectomy was studied.

Method. In one group of dogs the portal vein and inferior vena cava were divided below the liver and continuity re-established by end to end anastomosis crossing portal to systemic and caval to portal circulations. (Fig. 1). One month later 70% hepatectomy was performed. Hepatic venograms, obtained by injecting contrast material directly into the inferior vena cava, were made prior to hepatectomy to prove patency of the caval-hepatic anastomosis and to visualize the intrahepatic portal bed. Venography was repeated at intervals afterward to demonstrate regeneration *in vivo*. After periods of observation ranging from 35 to 60 days post-hepatectomy the dogs were sacrificed by exsanguination under nembutal anesthesia. Their livers were removed and weighed and the degree of regeneration calculated. The assumption was made, based upon dissections of normal livers, that a 70% hepatectomy

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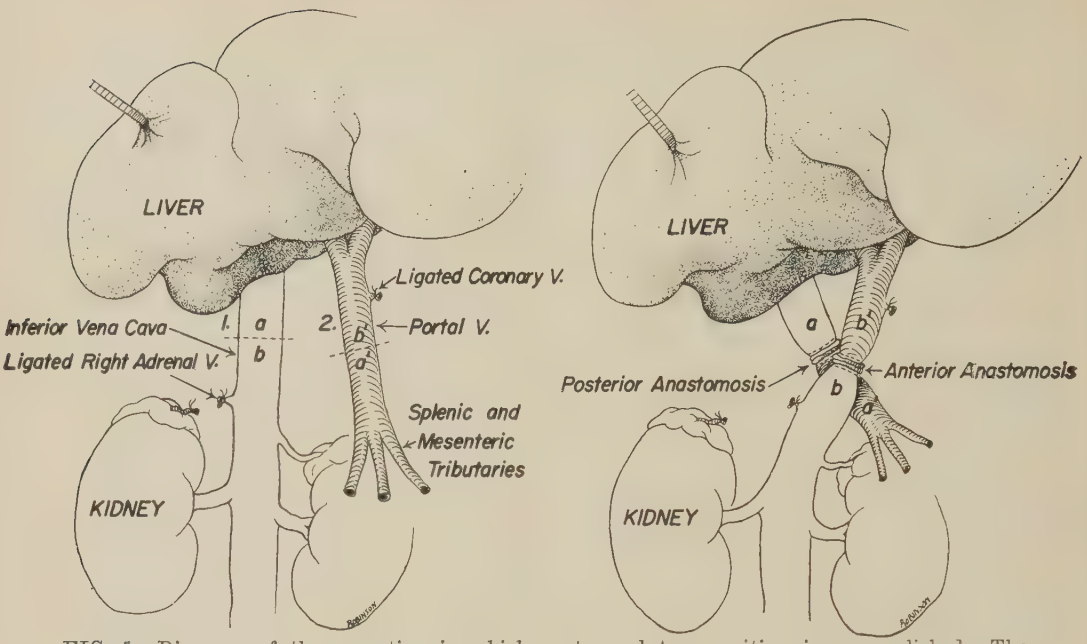

FIG. 1. Diagram of the operation in which portacaval transposition is accomplished. The inferior vena cava is divided at 1 and the portal vein at 2. The first anastomosis is performed between a and a′ and the second between b and b′.

had been performed. The weight, therefore, of the operative specimen was multiplied by 3/7 to obtain the weight of the hepatic remnant, which, when subtracted from the weight of the liver removed at autopsy gave an estimate of the increase in liver weight during the experimental period. The gain was divided by the weight of the liver removed at operation and multiplied by 100 to express regeneration in per cent. Thus if gain in liver weight equalled the weight of liver removed at operation, regeneration would be 100%. Partial hepatectomy was also carried out in dogs with normal hepatic circulation and with end to side portacaval shunts. X-rays were not taken but similar calculations were made.

Results. Eight dogs survived portacaval transposition and partial hepatectomy without incident. In all regeneration observed in the venograms was striking. When the venograms made prior to partial hepatectomy were compared with those taken at intervals afterward it was clear that there was a great increase in the area and density of liver filled with contrast material. The main branches of the portal vein were enlarged. Regeneration ranged from 20 to 80% with an average of 50% (S. D. 21). (Table I).

Three normal dogs survived partial hepatectomy. Regeneration ranged from 53 to 114% with an average of 75% (S. D. 34).

Only one dog with an Eck fistula survived partial hepatectomy long enough to be considered comparable. Regeneration was not found.

Discussion. The assumption that liver regeneration has occurred is based upon two lines of evidence, the venograms and the calculations of per cent regeneration. The venograms show that the liver casts a larger and more dense shadow after partial hepatectomy than before. This is interpreted as indicative of hepatic regeneration. The calculations of per cent regeneration are less easily evaluated because of pitfalls inherent in the method. The estimate of gain in liver weight during the post-hepatectomy period is based upon the measured weights of liver removed at operation and at autopsy and the assumption that a 70% hepatectomy had been performed. In support of this assumption it was found in dissections of normal livers that the portion of liver resected constituted 71% of the total weight of the liver

TABLE I. Hepatic Regeneration Following Partial Hepatectomy in 8 Dogs with (A) Portacaval Transposition, (B) 3 Dogs Normal Hepatic Circulation, and (C) 1 Dog Eck Fistula.

Wt of dog at part. hepatect., kg	Wt of liver removed, g	Estim. wt of hep. remnant, g	Wt of dog at autopsy, kg	Wt of liver at autopsy, g	Estim. gain in liver wt, g	Interval between op. and autopsy, days	Liver regeneration, %
A. Portacaval transposition							
15.5	273	117	13.6	302	185	38	68
16.1	302	130	15.2	371	241	36	80
13.6	335	143	12.3	230	87	60	25
10.8	251	108	9.3	159	51	50	20
11.1	201	81	9.6	177	96	50	49
10.2	159	68	9.3	161	93	45	59
21.5	322	138	18.2	320	182	45	57
15.4	272	117	14.3	230	113	35	42
						Avg regeneration 50% ± S.D. 21	
B. Normal hepatic circulation							
11.4	279	120	10.4	285	165	42	59
12.3	348	147	12.9	322	185	57	53
13.9	208	89	15.0	337	238	30	114
						Avg regeneration 75% ± S.D. 34	
C. Eck fistula							
16.1	421	181	10.7	174	—7	29	0

(±S. D. 4). The liver removed at operation, however, contained an unknown quantity of blood whereas the liver removed at autopsy contained very little. The estimate, then, of the weight of liver left at operation must be unduly high and the weight of the postmortem specimen low by comparison. These considerations fix the error of gain and per cent regeneration on the low side but since each group of animals were similarly treated the error must be fairly constant.

The figures show that considerable regeneration did occur in the dogs with portacaval transposition though to a somewhat lesser degree than in the normal dogs. Whether this difference in response to partial hepatectomy is apparent or real must await the accumulation of more data. At this time the reasons for this discrepancy remain speculative.

The hepatic regeneration observed in the dogs with portacaval transposition is regarded as significant for it is well established that normal dogs(1), regenerate their livers completely after partial hepatectomy and dogs with Eck fistulae(2) do not. The ability of systemic venous blood to support liver regeneration has been demonstrated. The likelihood of the existence of a portal factor is thereby decreased but not excluded. The requirement of the dog's liver for venous blood in adequate quantity is nevertheless considered established. This is consistent with the observation that the portal vein supplies the liver in dogs with at least 70% of its total blood supply(3).

Summary. 1. Partial hepatectomy has been carried out in dogs with portacaval transposition, with normal hepatic blood supply, and with end to side portacaval shunts. 2. In dogs with portacaval transposition the hepatic venograms showed striking regeneration. 3. Percentile regeneration was somewhat less in these dogs than in the normal but greater than in the dogs with end to side portacaval shunts. 4. These data discount the importance of portal blood *per se* in regeneration of the liver and lend support to the concept that the failure of hepatic regeneration in dogs with Eck fistulae is due in part at least to lack of venous blood flow.

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Prolongation of Life in High-Leukemia AKR Mice by Cortisone.* (20094)

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This experiment is one of a series designed to further explore the mechanisms of action of hormones in cancer processes, especially in relation to cancer prevention and cancer control. The study here reported provides evidence that chronic cortisone administration prolongs the life of female mice of the high-leukemia strain AKR. The prolongation of life resulted from prevention of spontaneous lymphoid leukemia deaths at the early age typical of this strain.

Materials and methods. Female mice of the high-leukemia strain AKR were given prolonged treatment with cortisone acetate starting when the mice were 4 weeks of age. Thirty-seven mice, 20 treated and 17 control, were observed until their death from leukemia or other causes. The data are incomplete as 2 treated animals are still alive at 16 months. The calculations are based on a terminal date of 16.4 months. The data are still further modified by the deliberate sacrifice and autopsy of 6 treated mice at 14 months of age. All other mice were, insofar as possible, autopsied and gross and microscopic examination of leukemic and suspected leukemic lesions conducted at time of death. The *treated mice* were injected subcutaneously with 1 mg/day in 2 divided doses of an aqueous suspension of cortisone acetate (Merck). Injections were given for 3 consecutive days, at monthly intervals, earlier studies having indicated this to be the maximum dose tolerated well when given at monthly intervals. Diet consisted of Purina laboratory chow and water fed *ad libitum*. All mice were bred.

Results. The mean length of life of the control females was 9.3 months and of the treated mice 14.3. The mean difference of 151 days was significant with $P < .001$. Fig. 1 shows the per cent mortality at different ages. Seventy-six per cent of the control animals had evidence of acute leukemia at death. Animals not exhibiting leukemia were only those unaccountably lost from the group. In the treated group, the usual macroscopic evidence of leukemia was absent. Histological evidence of mild infiltrating leukemia was found in a few animals. Often death seemed to be due to multiple infections and conditions related to cortisone treatment. Many bizarre lymphocytes, monocytes, and polymorphonuclear cells were seen in the older treated animals.

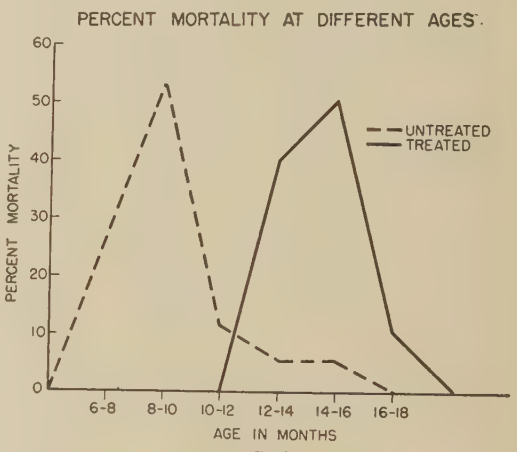

FIG. 1.

Discussion. The pronounced effects of pituitary adrenotropic hormone and adrenal cortical extracts on lymphoid tissues have long been known(1,2). A recent review of this subject is available(3). The effects of adrenal cortical and adrenotropic hormones on primary and transplanted lymphomas and leukemias indicate a possible relationship of the adrenals to the growth of these tumors(4-10). Recently, cortisone has been shown to inhibit significantly lymphoma development after x-radiation(11). Four cortical steroids were observed to be especially active in reducing the rate of transplanted lymphoid tumor growth in the mouse(12,13). In the present experiment cortisone was used because of its well-known ability to regress lymphoid tissues and,

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temporarily, lymphoid tumors. In selecting the high-leukemia AKR strain, consideration was given to the fact that in this case precancerous or early cancer changes would not have to be detected since leukemia at a relatively uniform age is an almost constant occurrence. The method of treating the animals periodically was chosen in an attempt to minimize the possible selection of cortisone resistant cell types as therapy proceeded. Preliminary tests indicated that 1 mg/day for 3 days was the maximum which could be used monthly with adequate survival. Prolongation of life with a decrease in the incidence of spontaneous leukemia has previously been reported as the result of (a) drastic caloric restriction in AK mice where the mean length of life was prolonged from 9.6 months to 14.0 months(14) and (b) total thymectomy at early ages in AK, RIL, and C58 strains where the mean length of life was prolonged from 7.6 to 12.7 months, 9.5 to 13.6, and 10.6 to 15.5 months respectively(15,16). The possible reasons for increase in longevity and reduction in occurrence of acute leukemia at early ages in the present study will be discussed in a later report.

Summary. Periodic administration of cortisone throughout life to high-leukemia AKR mice significantly prolonged their mean length of life from 9.3 months to 14.3 months. Prolongation of life resulted from prevention of spontaneous leukemia deaths at the early age expected in this strain.

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Life Span of the Red Blood Cell in the Hypophysectomized Rat.* (20095)

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The fundamental defect leading to any anemia must be either a decrease in rate of production of red cells or a decreased life span of red cells in the circulation or a combination of the two. Evidence in favor of each of these possibilities has been given to explain the anemia which characteristically follows hypophysectomy. Hypoplasia of the bone marrow(1) and a decrease in reticulocytes in the peripheral blood after hypophysectomy (2) are strong, but not conclusive, evidence that the post-hypophysectomy anemia results from a decreased activity of the bone marrow in producing erythrocytes. Hemosiderosis of the spleen of hypophysectomized rats(3) and a normal reticulocyte response after bleeding hypophysectomized rats(4) have been given as evidence that the pituitary is concerned with the control of blood destruction rather

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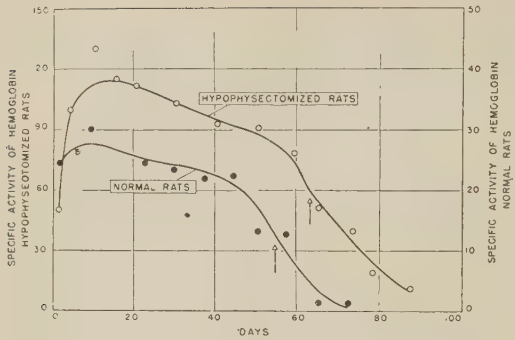

FIG. 1. Specific activity of the hemoglobin of hypophysectomized and normal rats in dis./min./mg $BaCO_3$.

than with blood formation. For this reason the present study using glycine-2-C^{14} to determine the life span of erythrocytes(5) in hypophysectomized rats was undertaken.

Method. Two groups of male rats of the Long-Evans strain were used in the experiment. One group was hypophysectomized at 40 days of age and given glycine-2-C^{14}, 48 days later. A group of normal controls of the same age was given labeled glycine at the same time. All animals were given 20 microcuries of glycine-2-C^{14} intraperitoneally. At frequent intervals blood samples were taken from a tail vein. In order to avoid excessive blood loss the animals were divided into groups of five, the samples of a given group being taken at two-week intervals. The specific activity of the hemoglobin was determined by methods previously published (6). The rats were weighed at weekly intervals and, since the normal group continued to gain weight, a correction for the increase in total red cell volume was applied (based upon evidence that, at this age, the total red cell volume per kg body weight remains constant(7)). Correction was not necessary for the hypophysectomized rats since their weight did not change during the experiment.

Results. Fig. 1 shows the average C^{14} specific activity of the hemoglobin from both the normal and hypophysectomized rats as a function of time. Since the specific activity of the hemoglobin of the hypophysectomized rats was approximately three times that of the normal rat and the normal rats were approximately three times as heavy as the hypophysectomized rats, both groups of animals incorporated glycine-2-C^{14} into hemoglobin to approximately the same degree. Exact interpretation of the type of curve obtained from the plotted data is difficult. However, if the point of inflection of the curve is assumed to indicate the mean life span(5), then the mean life span of the red cell in hypophysectomized rats (64 days) is, if anything, somewhat longer than in the normal controls (57 days). The points of inflection were obtained by graphical differentiation of the two curves and are indicated by arrows in Fig. 1.

Discussion. It is evident from the results that the mechanism for the development of anemia following hypophysectomy does not involve a decrease in the life span of the red blood cell, but rather that there must be decrease in the production rate of red blood cells. If after hypophysectomy the life span of the erythrocyte is not decreased and yet the number of cells circulating is reduced to 55% of normal(8), then it follows that the rate of production of red cells by the marrow after hypophysectomy is at most, only 55% of normal.

Conclusions. The life span of the red blood cell of the hypophysectomized rat is not less than that of the normal rat as measured by the use of glycine-2-C^{14}. Since the circulating red cell volume drops to 55% of normal after hypophysectomy, it must be assumed that the marrow of the hypophysectomized rat is producing, at most, only 55% of the normal number of erythrocytes.

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Relative Dehydrogenase Activity of Kidney and Liver Slices from Control and Tumor Mice.* (20096)

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In a previous study we had employed the *in vitro* reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to evaluate the dehydrogenase activity of liver and kidney slices in control and tumor bearing mice(1). The data obtained did not indicate any striking difference in the dehydrogenase activity of liver or kidney slices from tumor mice. In later work on this point and in a review of the aforementioned data it was noted that the kidney to liver ratio (K/L) of the endogenous dehydrogenase activity/mg tissue was lower in some strains of tumor bearing mice than in the controls of the same strain.

We therefore undertook an investigation of the K/L ratio in mice of different strains with and without spontaneous mammary tumors. The present findings indicate that the relative intensity of the dehydrogenase activity of kidney and liver slices tends to be maintained within a relatively narrow range in the groups studied. This K/L ratio is shifted in the majority of tumor mice of the CFW and A strains to a lower value. Such changes may occur in animals with very small tumors and does not appear to be related to inanition or cachexia. In addition, there appears to be a relationship between the K/L ratios in the control mice and the appearance of spontaneous tumors within the strain.

Materials and methods. The control mice included adult virgin females of the CFW, A, dba, and C3H strains. The tumor animals were female CFW, A, dba, and C3H mice bearing spontaneous mammary carcinomas. The animals were sacrificed by crushing the cervical cord and the kidneys and livers were quickly excised. Slices of approximately 1 mm in thickness were prepared from the left lobe of the liver and the mid-portion of the kidney. The kidney sections being sagittal included both cortical and medullary tissue. The tissue sections were placed in wide mouthed test tubes containing 3 ml of 1% TTC and 1 ml of 0.9% saline, buffered to pH 7.2. The tubes were placed in an incubator maintained at 37°C and subjected to continuous gentle agitation for one hour. Under these conditions the water soluble colorless TTC is reduced enzymatically to a red water insoluble formazan. At the end of this time the solutions were decanted and the reactions stopped by fixing the tissues in 10% neutral formalin. The formazan deposited in the tissues was then extracted with repeated washings of acetone and the total volume made up to 5 ml. The color intensity was determined in a Coleman spectrophotometer set at 470 mmu. These readings were converted to μg of TTC reduced by the use of a calibration curve obtained by the reduction of known amounts of TTC with crystals of sodium sulfide. The concentrations/ml obtained, which correspond to the values previously reported(1,2) were multiplied by the volume, 5 ml to yield the total amount of TTC reduced. The acetone dried tissues were weighed on a Roller-Smith microbalance (0-50 mg) and the μg TTC reduced/mg tissue was determined. All determinations were run in duplicate. These values are taken to be indicative of the endogenous dehydrogenase activity (R_O).

Results. In Table I we have listed the pertinent findings obtained. It will be noted that there is a tendency for the R_O value for the liver slices from the tumor animals to be somewhat greater than those of the control animals in the CFW, A, and dba strains. At the same time, the R_O values for the kidney slices from the tumor animals tended to be somewhat lower than those of the control animals in the CFW, A, and C3H strains. However, the magnitude of these changes is

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TABLE I. Endogenous Dehydrogenase Activity of Kidney and Liver Slices from Control and Tumor Mice of Different Strains.

Strain	No.	Body wt (g)	Liver R_o	Kidney R_o	R_o K/L	P†
CFW						
T*	40	35.0 ± 3.81	21.53 ± 3.66	31.71 ± 3.99	1.51 ± .29	<.01
C*	28	28.2 ± 5.21	18.15 ± 1.9	33.24 ± 2.94	1.84 ± .22	
A						
T	13	28.9 ± 3.78	22.2 ± 3.82	36.6 ± 2.24	1.70 ± .31	<.02
C	18	21.6 ± 2.86	19.3 ± 3.9	38.0 ± 4.9	2.01 ± .31	
dba						
T	13	28.3 ± 3.41	22.8 ± 3.8	39.8 ± 4.7	1.77 ± .24	>.05
C	23	22.3 ± 3.11	17.85 ± 2.8	34.85 ± 5.1	1.98 ± .33	
C3H						
T	30	29.4 ± 3.82	20.56 ± 3.5	33.89 ± 4.14	1.69 ± .35	>.57
C	32	23.9 ± 3.58	20.79 ± 3.39	35.12 ± 2.85	1.74 ± .34	

* T = Tumor. C = Control.

† P = Probability that difference in the K/L ratios between control and tumor group is due to chance variation.

far from decisive. On the other hand, more significant differences are found when a comparison is made of the K/L ratios obtained in the tumor and control mice of the CFW and A strains. The K/L values listed in Table I represent the average values and standard deviations of the ratios of the individual mice in the group. The range of these values in each group is in the form of a normal distribution curve. It will be noted that the K/L ratios of the tumor mice of the CFW and A strains are significantly lower than those of the control mice of the same strains. On the other hand, no such difference is observed between the tumor and control group of the C3H mice. In the case of the dba mice the difference in the K/L ratio between the control and tumor groups is just below the level usually considered statistically significant.

In order to determine whether the size of the tumor was a factor in the observed changes in the K/L ratio, a comparison was made of the relationship between the tumor size and the K/L ratio in the CFW tumor mice. This group was divided in two; those with tumors weighing less than 0.6 g and those with larger tumors. In the group with the smaller tumors the mean K/L ratio obtained in 22 mice was 1.49 ± 0.27. The mean value in 18 mice with larger tumors was found to be essentially the same, *viz.* 1.53 ± 0.32.

Since the tumor bearing mice are heavier than the controls it is pertinent to question whether the K/L ratios might vary with body weight rather than with the presence or absence of a tumor.

If the difference in body weight were the critical variable that accounted for differences in the K/L ratios between the tumor and control animals then one would expect to find an inverse relationship between the K/L ratio and the weight of the tumor animal. To test this possibility we have divided the CFW tumor animals into 2 groups: those weighing <35 g and those weighing >35 g. The mean weights and K/L ratios in these 2 groups were found to be: body weight 32.5 g, K/L ratio 1.42 and body weight 38.0 g, K/L ratio 1.69. It is thus evident that the K/L ratio does not vary inversely as the body weight.

Discussion. A variety of clinical and laboratory studies have suggested that alterations in liver function and enzymatic activity occur in tumor bearing animals and patients(3-5). In addition, changes in the enzymatic activity of the kidney in the tumor host have been reported in regard to d-amino acid oxidase and arginase(4). Further evidence of a change in kidney function in cancer patients is suggested by the report of Reifenstein *et al.*(6) on the response of cancer patients to ACTH. They found that as a group, cancer patients showed a normal response after ACTH in regard to the fall in circulating eosinophils and the rise in the urinary K/creatinine ratio. However, as a group they showed subnormal changes in regard to the expected rise in urinary P/creatinine and uric acid/creatinine ratios. It will

be noted that these findings reflect not only an adrenal defect but also an alteration in renal function. In view of the above observations on hepatic and renal function in the tumor host, it might be expected that decided changes in the dehydrogenase activity of these tissues would be found. While our data indicate a tendency for the dehydrogenase activity of the kidney slices to fall and the liver slices to rise in the tumor group as compared with the controls, these changes are of limited magnitude and the overlap of the absolute values in the 2 groups is considerable.

The present findings are of interest from several aspects: (a) In view of the importance of the homeostatic functions of the liver and kidney(7) and clinical observations linking kidney and liver function; *i.e.*, the hepatorenal syndrome, it is significant to find evidence of a relatively fixed relationship between the dehydrogenase activity of the liver and kidney. (b) The fall in the K/L ratio in the tumor mice of the CFW and A strains is indicative of a systemic alteration in the tumor host. Since this change was found in the mice with small as well as large tumors, it more probably represents an effect of the tumor rather than an expression of cachexia or inanition. (c) The findings are of interest in regard to the incidence of spontaneous tumors within the strains. The most marked differences in the K/L ratios between the control and tumor mice were found in the CFW and A strains. These strains are known to have a very low incidence of spontaneous breast tumors in the virgin state. In contrast, the K/L ratios in the control and tumor mice of the C3H strains were almost identical. This strain is characterized by a very high (90%) incidence of spontaneous tumors in the virgin mice. The dba strain has an intermediate incidence of breast tumors in the virgin females (11-50%). In this strain the difference in the K/L ratios between the control and tumor animals was found to be just below the level of statistical significance.

Summary. The *in vitro* dehydrogenase activity of liver and kidney slices from female control and tumor mice of the CFW, C3H, dba, and A strains was determined and the relative intensity of the kidney to liver values (K/L ratio) was calculated. The K/L ratio was found to be maintained within a relatively narrow range in the different control groups. In the CFW and A mice this value was shifted to a lower value in the presence of a tumor. Such a change occurred to a lesser degree in the dba mice and not at all in the C3H mice.

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A Plasminogen Activator in Spontaneously Active Human Blood.*† (20097)

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In the living organism fibrinolytic activity of blood occurs in a number of physiological and pathological conditions(1,2). The high activity found after certain modes of death (3) has recently been studied(4). The specific fibrinolytic effect observed in blood is caused by a strong adsorption of the fibrinolytic substances to fibrin(5). The experi-

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ments also revealed the presence of a plasminogen activator in the active samples.

Methods and materials. Fibrinolytic activity was measured by: 1) *The standard fibrin plate method*(6) in which the substrate contains a large amount of plasminogen. 2) *The heated fibrin plate method*(7) where the plasminogen in the substrate was destroyed by heating the fibrin plates at 85° for 45 minutes.

Fibrinolytically active globulin was obtained from active samples of human plasma by precipitation at pH 5.3 and low ionic strength(8) and redissolved in buffer to the original volume. *Normal human globulin* was prepared from serum in the same way, but dissolved to ½ of the original volume. *Human plasminogen* was precipitated from serum in the same way, redissolved and reprecipitated by dialysis against distilled water for 2 hours (mechanical stirring) and finally dissolved in buffer to 1/10 of the original volume. *Bovine plasminogen.* Freshly prepared bovine fibrinogen(6) was heated at 56° for 5 minutes in a waterbath. After cooling and centrifugation the supernatant was dialyzed against saturated ammonium sulphate, in the cold, overnight. The precipitate was redissolved, dialyzed against buffer and lyophilized. *Buffer.* Sodium diethylbarbiturate buffer (Michaelis) pH 7.75, containing sodium chloride to an ionic strength of 0.15. *Streptokinase.* A sample was kindly placed at our disposal by Dr. Frank Fletcher, Cheshire, England.

Results. Blood from cases of sudden death. Active blood samples from cases of sudden anoxaemic death(4) contain no fibrinogen or fibrin. The activity of the globulins from 13 different cases was compared by means of the two methods. High activities were found on standard fibrin plates (containing plasminogen), while very low activities were found on heated fibrin plates (containing no plasminogen). Addition of plasminogen to the solution of active globulin produced a large increase in activity on heated fibrin plates. On standard fibrin plates a smaller increase was obtained (Fig. 1). These experiments show that the active samples contain a plasminogen activator. Various pro-

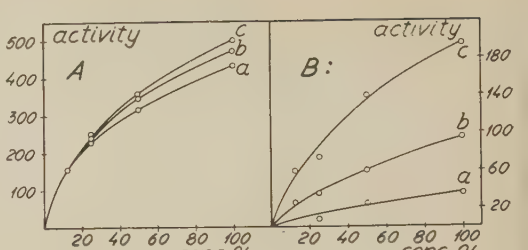

FIG. 1. Dilution curves of mixtures of: a) active globulin and buffer, b) active globulin and human plasminogen and c) active globulin and bovine plasminogen. *A:* Standard fibrin plates (containing plasminogen) *B:* Heated fibrin plates (without plasminogen). *Abscissa:* Relative concentrations of mixtures in %. *Ordinate:* Activity as product of 2 diameters of the lysed zones (mm^2).

teases produce almost identical effects on the standard fibrin plate and on the heated fibrin plate(7). Therefore the effect obtained on standard fibrin plates is caused primarily by activation of the plasminogen contained in the substrate. An estimation of the content of activator in active globulin was obtained on heated fibrin plates as follows: An excess of bovine (A.1) and human (A.2) plasminogen was added to a solution of active globulin and the resulting activities were compared with the enzyme activity (plasmin) of the active globulin (B) and with the activity, which could be produced by maximal activation of normal human globulin by addition of active globulin (C.1) or of streptokinase (C.2) (Table I). The results show that the addition of bovine plasminogen (A.1) produced an amount of plasmin which was about 40 times larger than the amount contained in the original sample of active globulin (B) and about 20 times larger than the maximal amount which could be produced by activation of the sample of normal human globulin. Identical activities were obtained by activation of human globulin with active globulin (C.1) or with streptokinase (C.2). The relatively lower activities obtained by addition of human instead of bovine plasminogen to the active globulin was caused by contamination of the preparation with inhibitory substances.

Blood from living persons. Samples of active globulin were also obtained from living persons under strain (electrically induced convulsions in psychiatric therapy). Previ-

TABLE I. Comparison between: (A) The Activity Produced by Addition of Plasminogen to Active Globulin; (B) the Activity Produced by Active Globulin Alone; and (C) the Activity Produced by Maximal Activation of Normal Human Globulin with Active Globulin (C. 1) and with Streptokinase (C. 2).

	Mixtures	ml	Measured activities of dilutions 1	½	¼	⅛	1/16	Activities as % of max. activity Uncorrected	Corrected
A. 1	Active globulin	.50							
	Bovine plasminogen	1	196	146	97	64	49	100	400
	Buffer	.50							
A. 2	Active globulin	.50							
	Human plasminogen	1	144	78	56	44	—	52	208
	Buffer	.50							
B.	Active globulin alone		65	49	25	19	—	11	11
C. 1	Human globulin	1							
	Active globulin	.50	94	64	40	32	—	20	20
	Buffer	.50							
C. 2	Human globulin	1							
	Streptokinase	1	81	64	49	36	—	20	20

Substrate: Heated fibrin plates. *Measured activities:* Products of 2 diameters of the lyzed zones (mean of 3 determinations) in mm². *Activities calculated as % of max. activity:* Uncorrected values are obtained by interpolation on curves drawn from measured activities (compare Fig. 1). These percentages are corrected for dilution in order to compare the activities which correspond to plasma concentrations of active globulin and of human globulin. For concentrations of active globulin (A. 1, A. 2, B) and human globulin (C. 1, C. 2) see: Methods and materials. The concentrations of bovine plasminogen (in A. 1), human plasminogen (in A. 2), active globulin (in C. 1) and streptokinase (in C. 2) necessary to yield maximal activities were estimated in separate experiments.

ous experiments had shown that the fibrinolytic substances in blood were strongly adsorbed to fibrin(5). Thus globulins prepared from serum obtained from active blood were completely inactive, while globulins prepared from the oxalated plasma were fibrinolytically active. Even the slightest clotting in the globulin solutions gave erroneous results, when the remaining solution was used for the estimation of fibrinolytic activity. The high lability of fibrinogen in the plasma globulin solutions greatly increased this difficulty and made the fibrin plate method a very suitable method. The amount of activator in plasma globulins from living persons under strain in 10 different cases was determined as described above. A 2-3 times increase in the amount of plasmin could generally be produced by addition of bovine plasminogen to active globulin solutions.

The activator-plasminogen reaction. In some experiments the activities of mixtures of plasminogen with active globulin (from cases of sudden death) were also measured by means of lysis time determinations on bovine fibrin, performed as described in (9). Solutions of active globulin containing appropriate concentrations of human a) and bovine b) plasminogen were left for 15 min. at 37°C. Fibrinogen was added and clotted with thrombin and the lysis times were determined (Fig. 2). Increasing amounts of plasmin were formed by addition of increasing amounts of plasminogen to the active globulin. Similar results were obtained by means of the heated fibrin plate method.

These experiments indicated that the activation was probably a stoicheiometrical reaction. However, the bovine fibrin used is not a suitable substrate to settle this question because of its high plasminogen content. Furthermore the experiments were complicated by the instability of the activator and by a significant content of inhibitory substances in the plasminogen and activator preparations.

Discussion. It is generally accepted that plasmin is produced in blood by activation of a precursor, plasminogen(1). In the human organism fibrinolytic activity is known to develop under various physiological and pathological conditions(1,2), but no conclusive evidence has been presented concerning the

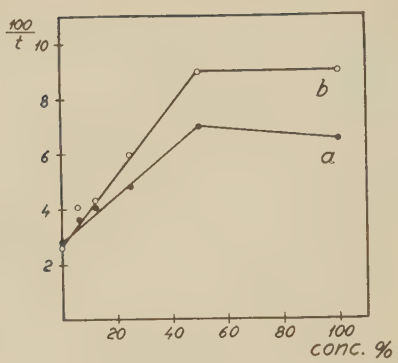

FIG. 2. Lysis times of clotted mixtures of: Active globulin (1.0 ml); thrombin solution (1.0 ml); plasminogen (1.0 ml), and bovine fibrinogen (.05%; 1.0 ml). The mixtures were incubated at 37°C for 15 min. before addition of fibrinogen. *a:* Human and *b:* bovine plasminogen added in increasing concentrations. *Abscissa:* plasminogen concentrations in % of stock solution. *Ordinate:* reciprocal lysis times.

mechanism involved. *Astrup and Permin*(10) demonstrated a physiological tissue activator, fibrinokinase, which later was found to activate plasminogen by a stoicheiometrical reaction(11). An interaction between this factor and the plasminogen in blood has been suggested as a possible explanation(10,1,3,4). Some authors have suggested the presence of an activating principle in blood. *Schmitz* (12) obtained two fractions from dried horse fibrin. Each was slightly active, while an increase in activity was produced after mixing. *Lewis and Ferguson*(13) prepared two fractions from normal dog blood serum. Each was inactive, while increasing activity developed after a period of several weeks, when they were incubated together at 5°C.

In the present work a plasminogen activator is demonstrated in spontaneously active human blood. The amounts present far exceed those necessary for the complete conversion of the plasminogen contained in blood. The activator is soluble and appears rather labile. A similar soluble and labile activator was recently isolated from urine(14). The tissue activator is more stable and is very strongly attached to structural proteins. It is known, furthermore, that fibrinolytic activity develops very suddenly and appears overall in the blood(1). Therefore, the blood activator is probably not identical with the tissue activator. There is reason to assume that the blood activator develops from a precursor, a proactivator, which apparently is present in large amounts in human blood.

Obviously, the determination of fibrinolytic activity yields different results with different substrates. A substrate free from plasminogen reacts only with the enzyme proper (plasmin). In a fibrinolytic method, performed as is normally the case, the plasminogen in the substrate may be activated by activators present in the active samples. Therefore, the total activity observed may be much larger than that corresponding to the content of plasmin in the sample. This makes a reconsideration of a large part of the existing literature in this field necessary. *Macfarlane and Pilling*(15) diluted active human globulin with *human fibrinogen containing plasminogen* and lysis was obtained in high dilutions. These large activities do not conform with the low spontaneous activities (plasmin) found in human blood with casein(8). *Lewis and Ferguson* have used bovine fibrin as a substrate(13). This substrate contains plasminogen and does not permit a distinction between activator and plasmin. Our recent experiments suggest that it is an activator and not an enzyme, which is formed spontaneously in their cited experiment.

Summary. 1. Fibrinolytically active blood, as obtained from the living and dead human organism, contains a plasminogen activator. The amount of this activator exceeds many times that needed for the complete activation of the plasminogen present in human blood. 2. The plasminogen activator in blood differs from the tissue activator, fibrinokinase, and is probably formed from a precursor contained in the blood.

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Effect of Chlorides and Heat on Extraction and Activity of Sheep Pituitary Gonadotrophin.* (20098)

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Aqueous solutions of organic and inorganic compounds, and also water have been used for the extraction of pituitary gonadotrophin(s)(1-5). The concentration and, particularly, the separation in high yields of two biologically pure gonadotrophins from these extracts have been difficult to accomplish. Presumably this is due in part to the association of the gonadotrophins (FSH and LH) with inert material and with each other. On the basis of this assumption attempts were made to dissociate these hormones by extracting them with saturated solutions of inorganic chlorides, concentrated solutions of sucrose and urea, and water at a temperature just below that which would cause inactivation. Although separation of the gonadotrophins was not accomplished, they were concentrated by simple procedures and information was obtained relative to their stability to heat and their augmentation by concentrated salt solutions.

Methods and materials. The solvents used for extracting the dry sheep pituitary tissue were solutions of inorganic chlorides saturated at room temperature, 0.88 M sucrose, 6.6 M urea and water. The amounts of pituitary tissue extracted ranged from 2 to 100 g. Extractions with sodium chloride were made at 25, 60, 70, 80 and 90°C. Extractions with the other solutions were done at 70°C. The pituitary powder was mixed in the ratio of 1 g of dry tissue to 10 ml of solution, placed in a water bath regulated to the desired temperature, stirred for one hour, centrifuged and the residue reextracted in the same way. The two extracts were combined and designated as extract E. A third treatment with NaCl solution extracted little activity. On the basis of this, the residues that remained after two extractions with the other solvents were not studied. *The E extracts* were dialyzed to remove the chlorides and other substances used for extraction. This caused an inactive precipitate to form which was removed by centrifugation to give the dialyzed soluble fraction A which was adjusted to pH 4. The precipitate was removed by centrifugation and designated as fraction C, and the pH 4 soluble fraction (B) was concentrated by lyophilizing. This concentrated extract was dialyzed, then centrifuged to remove any insoluble material present and lyophilized to dryness to give fraction D. Fraction B was also recovered by precipitation with alcohol or acetone and dried by lyophilization. The *extracts and fractions* were assayed for gonadotrophic hormone activity by the use of 21-day-old female rats of the Holtzman-Rolfsmeyer strain. The volumes of the fractions were adjusted with

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TABLE I. Gonadotrophic Activity of Fractions from Saturated Sodium Chloride Extracts of Sheep Pituitary Glands.

Fractions*	Dose, mg eq.†	Temp. of extraction, °C: 25	60	70	80	90	Protein (N × 6.25), mg/g eq. at 60°C
		Avg wt of ovaries, mg					
E	100	269±18.3 (7)‡	205±16.0 (5)	194(1)			
A	100	88±11.2(13)	93± 7.2(12)	101±11.8(6)	48(3)	17(6)	24.0(2)
B	100	107± 8.8(15)	97±12.7(12)	116(3)	24(3)	17(4)	12.8(2)
C	500	16± 0.9 (9)	15(3)				

* Extracts and fractions given in this and following tables are designated as follows: Undialyzed saturated extract, E; dialyzed soluble, A; dialyzed pH 4 soluble, B; and pH 4 insoluble, C. The pH 4 soluble B fractions were concentrated and are designated as D fractions in subsequent tables. The insoluble fraction that separated on dialysis was inactive. A total dose of 100 mg produced ovaries that averaged 14 mg, and 500 mg produced ovaries averaging 17 mg as compared with 13 mg for ovaries from uninjected rats of same age.

† Material equivalent to the mg of dry pituitary tissue indicated in this and subsequent tables.

‡ Figures given in parentheses following the stand. errors of the mean ovarian weights in this and other tables indicate the No. of rats used for assaying the various fractions.

normal saline so that each rat received the proper equivalent of dry pituitary tissue in nine injections of 0.5 ml each. The first injection was made on the afternoon of the first day followed by injections on the morning and afternoon of each of the next four days. Autopsy was performed on the morning following the last injection. The ovaries were removed, dissected free of other tissues, and weighed. The presence of follicles and corpora lutea was recorded.

Results and discussion. Sodium chloride. The results of the gonadotrophic assays of the fractions obtained from the concentrated NaCl extracts are given in Table I. The A and B fractions obtained at 25, 60 and 70°C had essentially the same activity; fraction C contained little activity. The gonadotrophin was stable when heated at 70°C in saturated NaCl solution, whereas at 80°C the hormone was partially inactivated and almost completely inactivated at 90°C. On the basis of these results extractions with concentrated solutions of other compounds were done at 70°C.

Potassium chloride. Saturated KCl extracts prepared at 70°C were fractionated and the results of the assays are summarized in Table II. The D fractions recovered from the NaCl and KCl extracts contained both follicle stimulating and luteinizing activities indicating that a separation was not accomplished by the procedures of extraction and fractionation given above. Fraction D obtained from the KCl extracts was not inactivated when dissolved in saturated KCl and heated a second time at 70°C for one hour followed by dialysis against 0.5% KCl. A total dose of 100 mg of this fraction produced ovaries that had an average weight of 100 mg before, as compared with 102 mg after, the second heat treatment.

TABLE II. Gonadotrophic Activity of Fractions from Saturated Potassium Chloride Extracts of Sheep Pituitary Tissue.

Fractions*	Dose, mg eq.	Avg ovarian wt, mg	Protein (N × 6.25), mg/g eq.
E†	100	201± 8.1 (5)	
A	100	104± 5.7(41)	40.9±2.3(8)‡
B	100	102± 7.9(27)	16.4±1.2(9)
C	250	29±10.0 (4)	
C	500	59±11.0 (9)	28.9±2.6(7)
D	100	104± 9.4(13)	10.5±1.2(4)

* Extracts were made by heating at 70°C.

† Letters have same meaning as given in footnote 1 of Table I.

‡ No. of preparations on which avg protein values are based.

Barium, calcium, magnesium and lithium chlorides. The fractions obtained by dialysis of the extracts made with saturated solutions of these chlorides were less active than the corresponding ones obtained from NaCl and KCl extracts. The $BaCl_2$ fraction was the most active of the 4, the LiCl one was essentially inactive. The ovaries produced by the $BaCl_2$ fraction consisted mainly of follicles which may bear some relation to the fact that the pH of the $BaCl_2$ solution was 4.9

TABLE III. Gonadotrophic Activity of Aqueous, Sucrose and Urea Extracts of Sheep Pituitary Tissue.

		Kind of extract				
		Water		Sucrose (0.88 M)		
Fraction*	Dose, mg eq.	Ovarian wt, mg	Protein, mg/g eq.	Ovarian wt, mg	Protein, mg/g eq.	Urea (6.6 M), ovarian wt, mg
E†	100	112± 8.4(9)	93.7(2)			
A	100			142±13.4(6)	51.0(2)	21(3)
A	500					178(3)
B	100	127±10.7(9)	62.5(2)	132±10.5(6)	33.4(1)	
C	500	21(3)		25(3)		
D	100	113±10.3(6)	25.8(2)			

* Extractions made at 70°C.
† Letters have same meaning as in footnote 1, Table I.

TABLE IV. Augmentation of Sheep Pituitary Gonadotrophin by Concentrated Solutions of Sodium Chloride and Sucrose.

Fraction	Dose, mg eq.	Fraction dissolved in:	No. of rats	Avg ovarian wt, mg	Protein, mg/g eq.
KCl (S30A)	100	.9% NaCl	10	107	12.3
		.5 sat. NaCl	4	159	
Aqueous (P89)	100	.9% NaCl	9	106	40.2
		.5 sat. NaCl	6	235	
		.44 M sucrose	3	150	
		.88 M "	3	137	

as compared with 6.9 and 7.1, respectively, for the saturated NaCl and KCl solutions.

Water, sucrose and urea. The results given in Table III show that a major part of the sheep pituitary gonadotrophin was extracted with water at 70°C. The aqueous extract E and fraction B had essentially the same activity, and the latter fraction was concentrated without loss of activity to give fraction D. Fraction C contained little activity. Sheep pituitary gonadotrophin was also extracted at 70°C with 0.88 M sucrose. This solvent removed less protein from the dry tissue than did water. Fraction A obtained from the 6.6 M urea extract contained little gonadotrophin.

Augmentation of pituitary gonadotrophin by concentrated salt solutions. The undialyzed extract E obtained with saturated NaCl at 25°C produced ovaries that had an average weight of 269 mg as compared with 88 mg for the soluble fraction A obtained from extract E by dialysis. This effect was also obtained with NaCl extracts made at 60 and 70°C (Table I), and with saturated KCl extracts made at 70°C (Table II). These saturated extracts were diluted about 1:1 with 0.9% NaCl to give approximately 0.5 saturated solutions for injection purposes. This concentration of NaCl and KCl was toxic. Results given in Table IV were obtained by the administration of two gonadotrophic preparations (P89 and S30A) in 0.9% NaCl and 0.5 saturated NaCl solutions. These preparations were equivalent in activity when administered in 0.9% NaCl, but P89 which contained over three times as much protein as S30A was more effectively augmented by 0.5 saturated NaCl than was S30A. These results show that the degree of augmentation produced by concentrated NaCl is influenced by the protein content of the preparation. The protein contents (N x 6.25) of the various fractions are given in Tables I, II, III and IV. Slight augmentation occurred when P89 was administered in 0.44 M and 0.88 M sucrose solutions. These data support the hypothesis that the augmentation of gonadotrophin by concentrated NaCl and sucrose solutions is due to the slow release of the hormone from the injection site. This explanation has been used to explain the augmentation of gonadotrophin by non-specific substances such as heme(6) and insoluble metallic hydroxides(7).

Summary. Concentrated fractions of dry

sheep pituitary tissue obtained from extracts made at 70°C with water, saturated solutions of NaCl and KCl, and with 0.88 M sucrose contained the major part of the gonadotrophic activity. Fractions obtained from extracts made with saturated solutions of barium, calcium and magnesium chlorides, and 6.6 M urea at 70°C were much less active. Dialyzed soluble fractions obtained from saturated NaCl extracts made at 80°C contained little activity and those from extracts made at 90°C were essentially inactive. Extraction at 70°C with the saturated solutions and fractionation of the dialyzed extracts did not separate FSH from LH. The activity of the gonadotrophin was augmented when administered in solutions which were 0.5 saturated with NaCl and KCl.

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Naturally Occurring Fibromas of Grey Squirrels Related to Shope's Rabbit Fibroma. (20099)

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Six grey squirrels (*Sciurus carolinensis*) collected within a year in Maryland counties adjacent to the District of Columbia were covered with multiple tumors which in gross appearance, histologic structure, and on immunological grounds show close relationship to the fibromas originally described by Shope (1) in cottontail rabbits. Afflicted squirrels, which weighed around 300 g or less, appeared to be juveniles. The squirrel fibromas measured from a few mm to 2.5 cm in diameter and varied in number from 5 on one squirrel to between 50 and 100 on others. They were located indiscriminately on all parts of the animals from eyelids to tail and toes. A squirrel with naturally occurring tumors is shown in Fig. 1. As discussed below, woodchucks (*Marmota monax*) were experimentally infected.

Materials and methods. Fibromas when harvested for passage were minced with scissors and ground with sand before being made into 10% emulsions with meat infusion broth. Due to the presence of skin and hair follicles, especially in woodchuck and squirrel biopsies, such emulsions were often contaminated as originally prepared. Addition of penicillin and streptomycin, followed by centrifugation for ½ hour, however, appeared to eliminate contaminants as a factor of importance. In addition animals inoculated with woodchuck or squirrel preparations were given 150,000 units of penicillin in oil. Some of the woodchuck fibroma emulsions were free from contaminants when tested in whole meat broth and on blood agar slants. In *neutralization* tests, the OA strain of Shope rabbit fibroma* was diluted with meat infusion broth, so that after addition to equal volumes of undiluted sera, the final virus dilutions were 10^{-2}, 10^{-3}, and 10^{-4}. Such mixtures in amounts of 0.5

* Kindly supplied by Dr. David R. Ginder, Emory University, Atlanta, Ga.

FIG. 1. Naturally occurring fibromas on grey squirrel.

ml were inoculated intracutaneously in domestic rabbits after incubating not longer than ½ hour at room temperature. All sera were inactivated by heating at 56°C for ½ hour. Each neutralization test included a control consisting of a titration of OA virus run in the presence of normal rabbit serum, fibromas constantly appearing at 10^{-4} and less frequently at a dilution of 10^{-5}. In other neutralization tests, fibroma virus of squirrel origin was used in final dilutions of 1:20 or 1:40, as titers above 10^{-2} have not been obtained so far with these preparations. Results of neutralization tests were read 5 days after inoculation.

Pathology. Histologic sections of tumors recovered from grey squirrels are similar to sections of rabbit fibromas studied in this laboratory and previously described by Shope (1), Andrewes(2), Ahlstrom(3), and Hurst (4). The epidermal covering of the tumors reveals slight hyperkeratosis and occasionally crusting. Focal elongation of the rete pegs is distinct in many sections. Moderate numbers of epidermal cells reveal cytoplasmic vacuolation as well as large cytoplasmic acidophilic and fuchsinophilic inclusions (Fig. 2). They stain with thionine but are not metachromatic. In addition they are unaffected by ribonuclease digestion (crystalline ribonuclease in pH 6 phosphate buffer at 37°C for one hour) and are periodic acid Schiff negative. These inclusions are also noted in islands of epidermis isolated in the upper corium as well as in squamous cells of outer layers of the hair roots. In the corium, and often extending into subcutaneous tissue, circumscribed but non-encapsulated nodules comprised of fibrocytes are present. The fibroma cells are associated with moderate quantities of reticulum and collagen but scanty fibroglia. Cell nuclei are round to oval and frequently vesicular. A rare mitosis is evident. Cell cytoplasms are usually abundant. Intracytoplasmic inclusions (Fig. 3) ranging in size from that of a micrococcus to that of an erythrocyte are observed in fibroma cells after staining sections by the Heidenhain modification of the Mallory collagen method

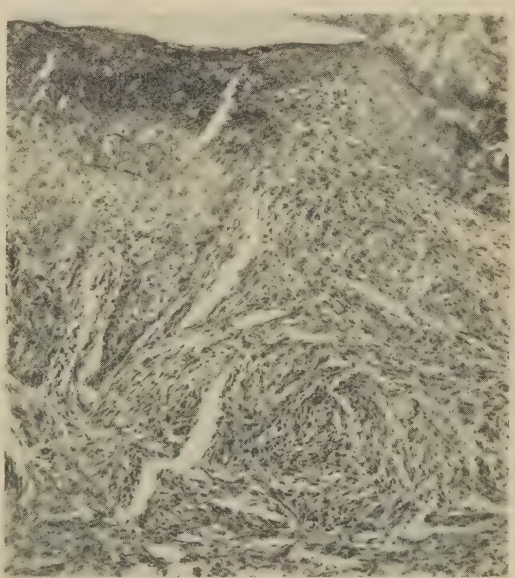

FIG. 2. Photomicrograph demonstrating intracytoplasmic inclusions in epidermal cells of elongated rete pegs at upper left, as well as portion of tumor. (H & E × 95.)

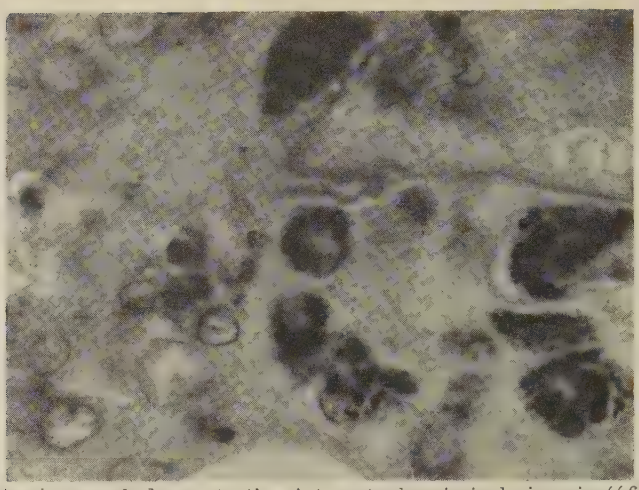

FIG. 3. Photomicrograph demonstrating intracytoplasmic inclusions in "fibroma" cell. (P.A.S. × 950.)

and the periodic acid Schiff procedures, but are not recognized after hematoxylin and eosin staining. These bodies fail to reveal any consistent distribution pattern within fibroma cells and are for the most part homogeneous. In several nodules there is a perivascular infiltrate of lymphocytes, plasma cells and histocytes involving blood vessels of the deep corium. In addition fibroma cells appear to radiate about vessels and to have had their origin from adventitial cells of these structures. Peripheral necrosis is occasionally noted.

Experimental. Transmission of squirrel fibromas was accomplished with difficulty at first, possibly because 5 of the squirrels with the original nodules had been dead for several days when received. Best results were obtained when fibromas from the single squirrel received alive were emulsified in 10% meat infusion broth and passed intracutaneously to a woodchuck. Four woodchuck passages have been carried out, the animals developing thick, plaque-like nodules under the skin which persist for 6 weeks or more. On inoculation into the skin of domestic rabbits, emulsions of woodchuck fibromas have led to the development of small nodules (1.3 cm in diameter) which appeared in 3 days and began to regress in 7 days (Fig. 4). Although growth of these fibromas was unaffected by the presence of normal squirrel or cottontail rabbit serum, their growth was completely or almost completely suppressed when squirrel fibroma-immune squirrel serum or Shope fibroma immune-cottontail serum was mixed in equal amounts with the original inoculum. Two consecutive passages of woodchuck fibromas have been carried out in domestic rabbits. Emulsions of woodchuck fibromas have also led to the formation of nodules on intracutaneous inoculation of grey squirrels. Initial attempts

FIG. 4. Tumors induced in domestic rabbit by intracutaneous inoculation of emulsion of woodchuck fibroma.

to pass the Shope rabbit fibroma to woodchucks and to squirrels have been unsuccessful.

Other experiments have further indicated immunological relationship between squirrel and Shope rabbit fibromas. In current studies 2 domestic rabbits were inoculated with fibroma emulsions, 2 inoculations having been given intracutaneously a week apart. The fibroma-emulsions were from 2 of the original squirrels, each rabbit receiving material from only one squirrel. When bled 10 days after the last inoculation, both rabbits had developed neutralizing antibodies against the OA strain of rabbit fibroma, as indicated by neutralization tests performed in the skin of a fresh rabbit. Preinoculation sera had no neutralizing capacity. Although one of the rabbits died, the second one failed to develop tumors when challenged with virulent OA fibroma virus. Only 2 serum specimens have been obtained from grey squirrels bearing tumors. Both of these sera had a definite capacity to neutralize OA fibroma in dilutions of 10^{-3} and 10^{-4}; whereas sera from 10 normal squirrels showed no neutralizing capacity.

Discussion. Although difficulties in obtaining grey squirrels and woodchucks for laboratory use have somewhat limited the extent of the investigations, our studies demonstrate a close relationship between squirrel and Shope rabbit fibromas. Further studies are in progress to determine how the agent of the squirrel tumor may be transmitted in nature, to define more clearly similarities and differences between it and the Shope fibroma virus, and to investigate the histochemical nature of cellular inclusions encountered.

Summary. Six grey squirrels collected in Maryland were found to have multiple small cutaneous nodules which histologically resembled Shope's rabbit fibroma. The squirrel fibroma was carried through 2 intracutaneous passages in grey squirrels, 4 consecutive passages in woodchucks, and from woodchucks for 2 passages in domestic rabbits. Cross-neutralization tests provided immunological evidence that the squirrel and Shope fibromas are related.

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Effect of Vitamin B_6 Deficiency on Hepatic Transaminase and Cysteine Desulfhydrase Systems. (20100)

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The relationship of vit. B_6 to transamination has been demonstrated in studies on animals(1-3) and microorganisms(4,5) deficient in this vitamin, and also in experiments with isolated enzyme systems(6-11). Schlenk and Snell(1) found that the tissues of vit. B_6-deficient rats exhibited decreased glutamic-aspartic transaminase activity. These findings were confirmed by Ames, Sarma and Elvehjem(2) and similar observations have been made by Shwartzman and Hift in studies on hamsters(3). *In vitro* experiments showed that pyridoxal phosphate activated resolved transaminase preparations(6-11) and recent evidence suggests that pyridoxamine phosphate may also function as a coenzyme for transaminase(12).

Previous communications from this laboratory(13-15) have described an hepatic transaminase system capable of catalyzing the following reaction: L-glutamine + α-keto acid $\rightarrow$ α-ketoglutaric acid + L-α-amino acid + NH_3. The available evidence indicates that a) a wide variety of α-keto acids may par-

ticipate in this reaction, b) the ammonia formed is derived from the amide group of glutamine, c) deamidation does not precede transamination, d) a similar reaction between asparagine and α-keto acids is also catalyzed by liver preparations, and e) the α-keto analogues of glutamine (α-ketoglutaramic acid) and asparagine (α-ketosuccinamic acid), possible intermediates in these reactions, are hydrolyzed to ammonia and the corresponding dicarboxylic α-keto acids by liver preparations. Although vit. B_6 has been implicated in the mechanism of a number of transaminase systems there is as yet no evidence that this vitamin or its phosphorylated forms is involved in the mechanism of the glutamine-α-keto acid transamination-deamidation reaction. We have therefore examined the livers of vit. B_6-deficient rats for this activity as well as for the classical glutamic-pyruvic transaminase activity. For comparative purposes studies have also been made on cysteine desulfhydrase since it has been reported that this system is reduced in activity in vit. B_6-deficient animals(16), and it is probable that pyridoxal phosphate is a coenzyme for cysteine desulfhydrase in *Proteus morganii*(17).

Experimental. Adult rats of the Buffalo strain(18) weighing 150 to 220 g were housed in individual cages and given water *ad libitum,* and the following vit. B_6 deficient diet* expressed in grams per kg of diet; Labco brand vitamin-free casein 180, cerelose 570, salt mixture 45(19), lard 200, choline chloride 5, and vitamin mixture 0.1. The vitamin mixture contained in grams per kg of completed diet folic acid 0.01, biotin 0.001, thiamine hydrochloride 0.04, riboflavin 0.02, niacin 0.2, and calcium pantothenate 0.14. Vit. A ester (10,000 units per kg of diet) and α-tocopherol (0.1 g) were dissolved in a portion of the lard and incorporated into the diet. In an effort to hasten development of the vit. B_6 deficiency syndrome(20), the rats were given 2.4 mg of desoxypyridoxine† (2,4-dimethyl-3-hydroxy - 5 - hydroxymethylpyridine) per week by intraperitoneal injection in 6 equal doses. All of the rats developed characteristic symptoms of vit. B_6 deficiency with severe dermatitis, scaliness of paws and moist snouts in 73 to 91 days, at which time they were sacrificed. The animals lost between 40 and 78 g (average weight loss = 57 g) during the experimental period. Control animals received the same diet supplemented with 25 mg pyridoxine hydrochloride per kg of diet. No significant differences in the levels of enzymatic activity were noted between control animals receiving the pyridoxine-supplemented diet and rats fed a stock purina chow diet. The animals were sacrificed by decapitation and exsanguination, and the livers were rapidly removed, chilled, and homogenized with 3 volumes of ice-cold distilled water. The determinations of transaminase activity were carried out as described previously(13,14). Cysteine desulfhydrase was determined according to Greenstein and Leuthardt(21). Pyruvate(22), glutamine, and glutamate(23) were determined as described. Crystalline pyridoxamine and pyridoxal phosphates were employed(24).

Results. The results, which are summarized in Table I, indicate that vit. B_6 deficiency, induced by dietary restriction of this vitamin and desoxypyridoxine injection, led to a marked decrease of cysteine desulfhydrase activity as indicated by considerably reduced formation of ammonia and pyruvate from cysteine. The lack of agreement between pyruvate and ammonia formation may be ascribed to further metabolism of pyruvate (25). The glutamic-pyruvic transaminase activity was reduced to about 40% of the control values, while the glutamine-pyruvic and the glutamine-phenylpyruvic reactions proceeded equally well in the livers of deficient and control animals. As indicated in Table I, addition of pyridoxal phosphate to the homogenates resulted in the restoration of most of the cysteine desulfhydrase activity. In several cases pyridoxamine phosphate was added and a comparable activation was observed. Conversion of pyridoxamine phosphate to pyridoxal phosphate would be expected to occur in crude liver preparations. Pyridoxal phosphate also increased the gluta-

* This diet is similar to one used previously in studies on mice(26).

† Generously supplied by Merck and Co.

TABLE I. Effect of Vitamin B_6 Deficiency on Several Hepatic Enzyme Systems.

Enzyme system	Deficient: No. of rats	Deficient: Range (μmoles)	Deficient: Avg	Control: No. of rats	Control: Range (μmoles)	Control: Avg
Glutamic-pyruvic transaminase*	14	2.27–3.10	2.64	14	5.35–7.09	6.36
reaction†	9	3.87–5.70	4.53	9	3.74–4.96	4.39
-phenylpyruvic reaction‡	14	4.88–7.50	6.41	14	5.24–7.45	6.22
-phenylpyruvic reaction§	10	5.52–6.76	6.22	10	6.00–7.14	6.44
Cysteine desulfhydrase; ‖ NH_3 formed	10	0 –1.44	0.39	10	2.18–3.60	2.89
Cysteine desulfhydrase; pyruvate formed	10	0 – .59	0.19	10	.88–3.02	1.38
Cysteine desulfhydrase; pyridoxal phosphate (100 γ) added; NH_3 formed	5	2.10–3.48	2.63	5	2.44–3.77	3.25
Cysteine desulfhydrase; pyridoxal phosphate (100 γ) added; pyruvate formed	5	1.08–1.36	1.16	5	1.38–2.28	1.78

* Reaction mixtures consisted of 1 cc homogenate (diluted 1:10), 1 cc 0.1 M veronal-acetate buffer (pH 7.2), 1 cc 0.01 M L-glutamate, and 1 cc 0.02 M sodium pyruvate; incubated 30 min. at 37°. Values expressed in terms of disappearance of glutamate.

† Reaction mixtures consisted of 1 cc homogenate, 1 cc 0.1 M veronal acetate buffer (pH 7.2), 1 cc 0.01 M L-glutamine, and 1 cc 0.02 M sodium pyruvate; incubated 4 hr at 37°; values given in terms of ammonia formation.

‡ Reaction mixtures consisted of 1 cc homogenate, 1 cc 0.1 M veronal-acetate buffer (pH 8.2), 1 cc 0.01 M L-glutamine, and 1 cc 0.01 M sodium phenylpyruvate; incubated 3 hr at 37°; values given in terms of ammonia formation.

§ Experimental details given in footnote ‡; values given in terms of glutamine disappearance.

‖ Reaction mixtures consisted of 2 cc homogenate, 1 cc 0.1 M potassium phosphate buffer (pH 7.5), and 1 u 0.03 M L-cysteine; incubated 2 hr at 37°.

mic-pyruvic activity of liver homogenates obtained from deficient rats, but not those of normal rats, a finding similar to that of Ames *et al.*(2) on the glutamic-aspartic system of vit. B_6-deficient rats.

Discussion. The present demonstration of reduced glutamic-pyruvic transaminase activity in liver homogenates of vit. B_6-deficient rats is in accordance with *in vitro* studies indicating the participation of pyridoxal phosphate in this reaction. Glutamic-pyruvic transaminase was reduced to approximately 40% of the control values in vit. B_6-deficient rats, which is about the same magnitude of effect observed for the glutamic-aspartic system in vit. B_6-deficient rats(2) and hamsters(3). The loss of cysteine desulfhydrase activity was striking and it should be noted that this activity could not even be demonstrated in the livers of 6 of the deficient animals studied. In the light of these findings the absence of a significant decrease in the activity of the glutamine-α-keto acid system in the deficient rats is of interest. This result may be interpreted as compatible with the concept that vit. B_6 is not involved in the glutamine-α-keto acid reaction, although the participation of vit. B_6 is not conclusively excluded. It is possible that the affinity of the glutamine system for vit. B_6 is greater than that of the other enzymes studied and that even under the severe conditions of vitamin deprivation and inhibition employed, sufficient coenzyme was retained for maximal activity.‡ Evidence derived from *in vitro* studies may be expected to aid in elucidating this problem.

Summary. 1. The glutamic-pyruvic, glutamine-pyruvic, and glutamine-phenylpyruvic reactions, and cysteine desulfhydrase activity were determined in homogenates of livers of vit. B_6-deficient rats and vit. B_6-supplemented control rats. 2. Glutamic-pyruvic transaminase activity was considerably reduced in livers of the deficient animals, while the glutamine-pyruvic and glutamine-phenylpyruvic reactions proceeded at approximately equal rates in control and deficient rats. 3. Cysteine desulfhydrase activity was markedly reduced in the livers of the deficient animals. Addition of pyridoxal phosphate to homogenates of the

‡ It is of interest in this connection that dialysis (24 hours against running water at 15°) of liver homogenates resulted in almost complete loss of cysteine desulfhydrase activity (almost completely reversed by addition of pyridoxal phosphate), while the glutamic-pyruvic and glutamine-α-keto acid systems were not significantly affected.

livers of the deficient animals resulted in restoration of most of the cysteine desulfhydrase activity. 4. The implications of these findings in terms of the participation of vit. B_6 in the glutamine-α-keto acid reactions are discussed.

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Response of Preputial and Adrenal Glands of the Rat to Sex Hormones.* (20101)

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Paired preputial glands are present in both male and female rats. They are considered accessory reproductive glands, since it is well known that they are stimulated by androgens and to some degree by other steroid hormones (1). Indeed, since they are well developed in both sexes, their weight has often been used as a measure of the endogenous androgen production of gonads or adrenal glands(2). Only recently has it become apparent that in certain aspects the response of the preputial glands to ACTH parallels that of the adrenal glands. Thus, highly purified preparations of ACTH are reported to exert a direct, growth-promoting action on the preputial glands of adrenalectomized-ovariectomized rats(3,4). Furthermore, such a growth response need not be attributed to a contaminating principle present in the ACTH preparation, since it has also been shown that preputial gland hypertrophy follows adrenalectomy, an operation known to evoke a hypersecretion of ACTH(5). It appears, therefore, that the direct action of ACTH is not limited to the cells of the adrenal cortex, but extends also to those of the preputial glands. Such a concept is in accord

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with the recent demonstration that either severe stress or intravenously administered ACTH produces a rapid fall in the level of preputial ascorbic acid(5), and with the fact that the preputial and adrenal glands show a similar response as regards weight and ascorbic acid level after hypophysectomy and following either acute or chronic administration of ACTH to hypophysectomized rats(6).

Much is already known about the effects of sex hormones on the weight of both of these glands but there is little information as to what effect these hormones have on the ascorbic acid content of either adrenal or preputial glands. It is currently thought that androgens cause a diminution in adrenal weight when given to rats of either sex, whether intact or gonadectomized(7). In contrast, estrogens induce adrenal hypertrophy in the rat, this effect being dependent upon the presence of the pituitary gland. Since both androgens and estrogens are believed to influence the weight of the preputial glands, it seemed of interest to make a comparative study of the effects of these hormones on the weight and ascorbic acid content of the preputial and adrenal glands. In addition, a study was made of the direct action of androgen treatment on the preputial and adrenal glands of the hypophysectomized rat as well as the effect of such a treatment on the responsiveness of these glands to the ascorbic acid-depleting action of ACTH.

Methods. Forty-five male rats of the Holtzman strain were divided into 3 equal groups when 30 days of age. All animals were kept in air conditioned quarters and given laboratory chow (Purina) and tap water. The animals in one group received daily, subcutaneous injections of 2.5 mg testosterone propionate, while those in the second experimental group were given subcutaneous injections of 50 μg estradiol benzoate daily. The third group served as untreated controls and all animals were killed 40 days after the injections were begun. In order to obviate any effects of pituitary stimulation the animals were quickly decapitated. The left adrenal and preputial glands were removed and after freeing them of fat and connective tissue, they were weighed on a torsion balance and their ascorbic acid

TABLE I. Effect of Estrogen and Androgen on Adrenal and Preputial Glands of Intact and Hypophysectomized Male Rats.

	No. rats	Adrenal glands				Preputial glands			
		Wt, mg		Ascorbic acid content, μg		Wt, mg		Ascorbic acid content, μg	
		Abs	Rel†	Abs‡	Rel§	Abs	Rel†	Abs‡	Rel§
Controls	13	18.2 ± .4¶	7.0 ± .2	82 ± 5.6	438 ± 21	54.4 ± 5.4	21.0 ± 2.2	29 ± 2.4	57 ± 3.4
Estrogen-treated	13	22.3 ± 1.0	14.0 ± .6	96 ± 6.1	388 ± 5.2	26.2 ± 1.9	16.5 ± 1.0	19 ± 2.2	81 ± 7.8
Androgen-treated	11	19.9 ± .6	8.8 ± .3	68 ± 6.0	342 ± 29	93.2 ± 13.0	42.0 ± 5.9	28 ± 3.4	33 ± 2.5
Hypophysect. (3 wk)	14	5.4 ± .2	5.1 ± .2	13 ± .6	247 ± 11	9.7 ± .5	9.1 ± .5	3 ± .9	24 ± 7.2
Hypophysect. (3 wk) + androgen*	17 L	6.1 ± .3	5.4 ± .2	11 ± .7	187 ± 14	42.4 ± 2.7	37.1 ± 2.2	6 ± .7	13 ± 1.5
	R‖	5.6 ± .3	5.0 ± .2	6 ± .8	106 ± 9.7	37.8 ± 3.4	33.1 ± 2.8	6 ± 1.0	16 ± 3.3

* 1.25 mg testosterone propionate inj. subcut. daily for 3 wk.
† mg/100 g body wt.
‡ μg ascorbic acid in entire gland.
§ μg ascorbic acid/100 mg tissue.
‖ 10 μg ACTH inj. intrav. 1 hr prior to autopsy.
¶ Standard error.

TABLE II. Effect of Estrogen and Androgen Treatment, Hypophysectomy and Hypophysectomy plus Androgen Treatment on Body, Testis and Prostate Weight of Rats.

	No. rats	Body wt, g		Left testis wt, mg		Ventral prostate wt, mg	
		Initial	Final	Abs	Rel†	Abs.	Rel†
Controls	13		260±6.6‡	1519±42	584±10.1	301 ±26	114 ± 1.0
Estrogen-treated	13		159±3.5	161± 7.8	103± 7.7	15.7± 1.0	9.2± .6
Androgen-treated	11		226±5.5	1237±25	549±15.4	856 ±43	378 ±16
Hypophysect. (3 wk)	14	122±1.7	109±1.8	206±12	191±11	9.5± .6	8.8± .5
Hypophysect. (3 wk) + androgen*	17	115±1.1	114±2.8	912±42	801±34	472 ±18	416 ±14

* 1.25 mg testosterone propionate inj. subcut. daily for 3 wk.
† mg/100 g body wt.
‡ Standard error.

content determined by the method of Roe and Kuether(8). Weights were also recorded for the left testis and ventral prostate of all animals. Thirty-one male rats of the Holtzman strain were hypophysectomized at the age of 33 days. These animals were maintained on laboratory chow and 5% sucrose solution during the post-operative period. Following hypophysectomy 17 of the animals were given daily, subcutaneous injections of 1.25 mg testosterone propionate. At the end of 3 weeks the left adrenal and preputial glands were surgically removed, using nembutal anesthesia. These glands were weighed and analyzed for ascorbic acid. Fourteen of these animals were then given an intravenous injection of 10 μg ACTH.‡ One hour following the ACTH injection, the animals were autopsied and the right adrenal and the right preputial glands removed, weighed and analyzed for ascorbic acid. As in the previous experiment the weight of the left testis and ventral prostate was recorded for each animal. The left adrenal and preputial glands were surgically removed from the remaining 14 animals at 3 weeks after hypophysectomy. The effect of an intravenous injection of ACTH on the ascorbic acid of the right glands was then determined. The results of this experiment have been previously reported(6) and only the values for the left glands are repeated here for the purpose of comparison. Testis and ventral prostate weights are likewise repeated for the animals in this group.

Results and discussion. The results of these experiments are summarized in Tables I and II. Due to the pronounced effect of the hormones on body weight, the weights of adrenal and preputial glands are recorded on a relative as well as on an absolute basis. For similar reasons, since gland weights were highly modified in certain groups, the ascorbic acid content has been calculated on both an absolute (μg in entire gland) and relative basis. This enables one to determine if the observed changes in the concentration of ascorbic acid are simply a reflection of the dilution or concentration effect imposed by hypertrophy or atrophy of the gland.

It is apparent that estrogen treatment causes a profound hypertrophy of the adrenal gland, the mean for this group being 100% more than that of the untreated, control animals. This is in agreement with previous reports in the literature(9-11) and according to present concepts is due to an increased secretion of ACTH. In direct contrast to this, we find that the preputial glands of the estrogen-treated group weigh much less than those of the control animals. Even on a relative basis, they are somewhat lighter, although when calculated in this manner the difference is not significant. In making these comparisons, it is well to bear in mind that the preputial glands are directly stimulated, not only by ACTH, but presumably also by androgens. In this regard, it is evident from the testis and ventral prostate weights of the estrogen-treated group that these animals are virtually hypophysectomized, insofar as gonadotropic hormone production and in turn androgen production is concerned. It is perhaps important, then, to notice that whereas the preputial glands are much reduced in weight in

‡ Armour ACTHAR (dosage expressed as LA-1A equivalent).

the estrogen-treated animals they are yet significantly heavier than those of animals which have been hypophysectomized for 3 weeks. It is conceivable that their weight represents the maximum that can be maintained over a long period of time by ACTH in the absence of androgen. This is in accord with the results obtained after treating hypophysectomized rats with ACTH(6).

The changes in the ascorbic acid content of the preputial and adrenal glands observed in the estrogen-treated animals can be largely accounted for in terms of the pronounced weight changes of these organs. Thus, the enlargement of the adrenal glands is accompanied by an increase in the total amount of ascorbic acid per gland. The latter is proportionately somewhat smaller than the increase in weight so that the concentration of ascorbic acid is less in the estrogen-treated than in the control adrenals. In contrast, there is a fall in the total amount of ascorbic acid in the preputial glands, but since this decline does not keep pace with the loss in weight, the concentration remains somewhat higher than in control glands. If one considers only the values for the concentration of ascorbic acid in these glands, it appears that estrogen treatment produces a reduction in the level of adrenal ascorbic acid but an increase in the level of preputial ascorbic acid. In such long-term experiments, however, such over-simplified interpretations seem unwarranted. Assuming that we are dealing with an estrogen-induced hypersecretion of ACTH, our results are compatible with those of Gordon(12) who found that continued daily injections of ACTH to rats for periods of several weeks was without effect on the level of their adrenal ascorbic acid.

It will be noted that the adrenal glands of the testosterone propionate-treated rats are heavier than those of the controls, either in terms of absolute or relative weight. This increase in adrenal weight is significant ($P < 0.02$). Although it is generally agreed that androgens inhibit the adrenal hypertrophy which follows castration of the male rat(13) data relating to the effect of androgen treatment on the adrenal weight of the intact male rat are fragmentary and completely unsatisfactory. The few papers which deal directly with this question indicate that androgen treatment is without any very striking effect on the adrenal weight of intact male rats (14-17). In some cases (as in the data of Korenchevsky and Hall, 1939) where it is possible to calculate relative adrenal weights a tendency for androgen treatment to produce adrenal hypertrophy is apparent. Our results leave no doubt that adrenal hypertrophy may result from androgen treatment although they do not preclude the possibility that under different experimental conditions (*i.e.*, factors such as dosage, length of treatment, age of animals, etc.) a qualitatively different outcome might result.

The literature is equally confusing in regard to the direct action of androgens on the adrenal glands of the hypophysectomized rat. Several workers have made the claim that when injections are begun at the time of hypophysectomy a partial maintenance of adrenal weight can be realized(18-21). Of the more recent papers on this subject only one by Lewis, DeMajo, and Rosemberg(22) reports a further diminution in adrenal weight as a result of androgen treatment. This effect was obtained in hypophysectomized female rats following the daily injection of either testosterone propionate (3 rats) or 17-vinyl testosterone (3 rats) for a 20-day period. The results obtained in the present study show that 1.25 mg testosterone propionate daily has no significant effect on the relative adrenal weight of the hypophysectomized male rat. It can be seen, however, that there appears to be a slight maintenance effect if only the absolute adrenal weight is considered, due to the tendency for the androgen treatment to prevent the loss in body weight otherwise seen after hypophysectomy. No doubt this general maintenance effect accounts, at least in part, for what has been regarded as a direct action of androgen on the adrenal. Thus, the data of Zizine, Simpson, and Evans(21) are much less striking if the adrenal weights are calculated on a relative basis. Even so, it is difficult to reconcile their results with those obtained in the present study. Analysis of their data indicates not only that androgen has a specific adrenal weight-maintaining

effect, but (contrary to their own conclusions) that this effect is at least in part dependent on the presence of the gonads. Since the dosage they employed was double that used in the present study, this may prove to be a valuable lead in the solution of this important problem. Turning now to the effect of androgens on the weight of the preputial glands, it is apparent that testosterone propionate is capable of producing marked hypertrophy of these glands in either the intact or hypophysectomized rat. This proves that androgen exerts a direct, growth-promoting action on the preputial glands although it is reasonable to suppose that in the intact animal androgens may also modify the size of these glands by virtue of their capacity to alter the rate of ACTH secretion. As a matter of fact, it seems likely that the estrogens as well as progesterone may affect preputial size chiefly in this manner. It is interesting to note that the relative size of the preputial glands as well as the ventral prostate is approximately the same in the intact and hypophysectomized androgen-treated groups, which further emphasizes the fact that the action of androgen on the preputial glands is predominantly a direct one.

It should also be pointed out that our results confirm previous reports(7) that androgen treatment will effectively maintain the weight of the testes of the hypophysectomized rat. Examination of histological sections of such testes shows the tubular epithelium to be essentially normal.

The situation with regard to the ascorbic acid in the adrenal and preputial glands is quite a different one than was seen after estrogen treatment. Thus, in considering the preputial gland, it is clear that androgens serve to enlarge the gland but do not produce a corresponding increase in ascorbic acid. This is seen in both the intact and hypophysectomized groups. By comparison, androgen treatment can apparently produce a diminution of adrenal ascorbic acid in the absence of any appreciable change in adrenal weight. This is seen in the intact groups where there is a significant difference in the concentration of ascorbic acid in the adrenals of the androgen-treated rats as compared with those of the controls. The same is true in the hypophysectomized groups where androgen treatment further reduces the already low level of adrenal ascorbic acid.

Finally, the effect of androgen treatment on the responsiveness of the adrenal and preputial glands to the ascorbic acid-depleting action of a single, intravenous injection of ACTH was determined. In a previous publication(6) it was pointed out that such an intravenous injection of ACTH would cause a precipitous fall in the level of ascorbic acid in the preputial and adrenal glands of the hypophysectomized rat. A comparable drop in the level of adrenal ascorbic acid can be observed in the present study at one hour after the intravenous injection of 10 μg ACTH into androgen-treated, hypophysectomized rats. In contrast, no such reduction in the level of the preputial ascorbic acid of these animals was observed. This indicates that testosterone propionate, in some manner, destroys the responsiveness of the preputial gland to the ascorbic acid-depleting action of ACTH.

Summary and conclusions. The administration of estrogen to intact male rats for 40 days induces hypertrophy of their adrenal glands but has the reverse effect on their preputial glands. The latter effect is undoubtedly a result of the virtual cessation of endogenous androgen production. Alterations in the ascorbic acid content of these glands appear to be a reflection of the marked changes in their weight. Androgen treatment for the same period produces a slight but significant increase in the weight of the adrenal glands and a 2-fold increase in preputial gland weight. This effect on the preputial glands is predominantly a direct one, as it is seen also in hypophysectomized animals. In contrast, no direct action of androgen on the weight of the adrenal glands of hypophysectomized rats was observed. It was found that androgen administration reduces the resting level of ascorbic acid in the adrenal and preputial glands of both intact and hypophysectomized rats. Furthermore, this hormone somehow destroys the responsiveness of the preputial glands to the ascorbic acid-depleting action of a single injection of ACTH.

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Angiostomy Cannulae for the Study of Pulmonary Circulation.*† (20102)

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London(1) used angiostomy cannulae for the purpose of directing hypodermic needles into the larger thoracic vessels. Daly(2) reported the measurement of pulmonary arterial pressure in the unanesthetized dog by means of the London Type cannulae. Johnson, Hamilton, Katz and Weinstein(3) in the same year studied the dynamics of pulmonary circulation by means of hypodermic manometers, the needles being placed in the aorta, pulmonary artery and pulmonary vein. Hamilton, Woodbury and Vogt(4) studied differential pressures in the lesser circulation of the unanesthetized dogs using angiostomy cannulae and hypodermic manometers. Katz and Steinitz(5) developed a modification of the angiostomy cannulae used by Hamilton, and used their cannulae in measuring pulmonary arterial pressures.

The purpose of this paper is to report the development of a technic permitting direct access to the atria and large thoracic vessels of unanesthetized dogs under conditions which may be compared to normal.

Methods. Two varieties of cannulae were developed and used. The first variety, shown in Fig. 1, a., consists of silver tubes (3 mm outside diameter) fitted with perforated silver plates, one type of which is trough-shaped, to fit over the left pulmonary artery. A second type is flat and oval in shape, suitable for attachment to the left atrium. The length of each silver tube is such that its distal end, the heart serving as reference point, will lie immediately under the skin of the chest wall. The perforated silver plates are made from 2 mm silver stock. The plate attachment that is fitted over the left pulmonary artery is 8 mm long and of sufficient width so that when bent in a semicircle of the correct radius it extends over half the circumference of the

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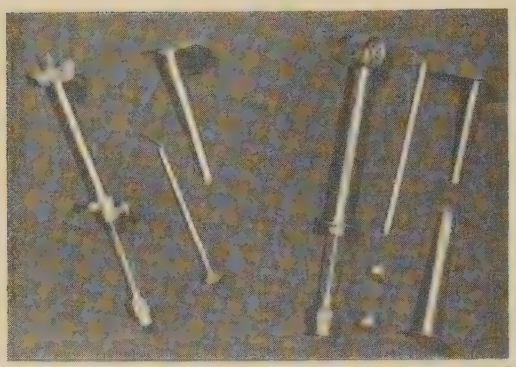

FIG. 1. a. Silver cannulae (left side). b. steel cannulae (right side) for surgical placement on left pulmonary artery and left atrium of dogs.

vessel. The oval plate for attachment to the left auricle measures 15 mm by 10 mm. The cannula tube is fixed in an opening 5 mm from one end of the oval plate. This arrangement allows placement of the cannulae on the auricle immediately beneath the entrance of the pulmonary veins into the atrium. Stainless steel stilettes must be used in the silver cannulae to prevent occluding fibrin formations and bending of the cannulae. These stilettes are made from 4 mm stainless steel rod; they are fitted on one end with No. 9 silverplated brass nuts. Hypodermic needles are fixed within the cannulae by means of "coupling" bushings equipped with 2 set-screws. These fittings are 7 mm long and of sufficient diameter so as to fit closely over the distal end of the cannula. One set-screw serves to fix the bushing to the cannula; the second set-screw serves rigidly to secure a hypodermic needle within the tube at the desired depth.

A *second type of cannula* shown in Fig. 1, b., is made of stainless steel tubing. Each cannula consists of 4 different parts: Two tubes, a proximal tube telescoping into a distal one; and 2 end pieces, one "closing" end piece and one "cannulating" end piece. *The proximal tube* measures 6.5 cm long and 3 mm in external diameter with a lumen of 1.5 mm. The proximal tubes are fitted with perforated plates 0.2 mm thick and are bent to conform in shape with the organ over which they are to be placed. One type is so shaped as to accommodate the left pulmonary artery and consists of a ring 10 mm in diameter and 4 mm wide. The width of the slit in the open ring is adjustable in order to allow it to slip around the artery when the vessel is collapsed. This open ring is fitted with a 10 mm length of polyethylene tubing of sufficient diameter to allow slipping the latter over the band portion of the ring so as to close the open portion when the device is placed around the left pulmonary artery. The tubing protects the vessel from possible trauma from the free edges of the slit in the steel ring. The second type of perforated plate, designed to attach to the left atrial wall, is oval, flat and flexible.

The distal tube of the cannula, which telescopes over the proximal tube, is formed by a stainless steel tube 6 cm long and 4 mm in external diameter. Its distal end presents a circular perforated flange for attachment to the chest wall. This arrangement enables instantaneous adjustment of the length of the cannula to the variations in depth of the pulmonary artery or left atrium from the skin surface. The inner aspect of the flange end of the distal cannula is threaded to the depth of 4 mm so that either of two end pieces, a "closing" or a "cannulating" end piece, may be screwed into it.

The "closing" end piece is a large headed screw which threads into the flange and allows complete closure of the cannula when not in use.

The "cannulating" end piece is a bushing threaded to fit into the flange of the distal cannula. It is perforated in its center so that hypodermic needles up to 14 gauge in size may be inserted, and is fitted with a small set-screw which enables the fixing of the needle within the cannula. It is necessary to use lengths of suitably-sized polyethylene tubing as stilettes for these cannulae, to prevent fibrin occlusion.

The cannulae are placed about the left pulmonary artery and attached to the left auricle through a thoractomy incision made in the left 4th intercostal space. Nembutal anesthesia is used, and respiration is maintained by an electric mechanical respirator. The pericardial sac over the left pulmonary artery is incised, thereby exposing this vessel and the adjacent left auricle. The visceral

layer of the pericardium over the left pulmonary artery is incised and freed from the vessel for a sufficient distance to allow the placement of the cannula over or around the vessel, depending upon the type of cannula used. After placement, the visceral pericardium is sutured over the cannula end piece. The oval atrial cannula plate is sutured to the muscular wall of the left auricle. Care must be exercised to avoid entering the chamber of the organ. Aqueous penicillin (10000 units) is injected into the pericardial and pleural sacs, after which they are closed with 4-0 silk suture. It is important that the atrial cannula be directed cranially and dorsally from its attachment and that the pulmonary artery cannula be directed laterally. In event the steel telescoping cannulae are used, it is necessary at this time to place the distal portions of these cannulae over the proximal portions. Following the placement of the above-mentioned stilettes within the tubes, the distal ends of the cannulae, either steel or silver, are then fixed to the chest wall in the subcutaneous fascia. The thoractomy incision is closed in three layers; the residual air in the pleural cavities is then removed by aspiration. The skin and subcutaneous fascia are sutured over the ends of the cannulae. Procaine penicillin (150,000 units) is administered intramuscularly.

Dogs are ready for use one week following surgical preparation. Prior to experimental study, the skin over the cannulae is anesthetized with 2% procaine hydrochloride solution. Incisions through the skin and superficial subcutaneous tissues are made to expose the distal ends of the cannulae. In the event steel cannulae have been used, the closing end pieces and polyethylene tubings are removed, and the "cannulating" end pieces are screwed into place. If silver cannulae have been used, the steel stilettes are removed and the "coupling" bushings fitted and fastened on the ends of the silver tubes. The exact distances from the distal ends of the cannulae to the left atrial cavity and to the pulmonary artery lumen may be determined by slowly inserting through the cannulae 20 gauge, 8 cm long needles fitted with saline-filled syringes to which negative pressure has been applied. The depth at which blood first appears in the syringe, indicates the desired position of the tip of the needle in the artery or atrium; this depth is marked on the needle with a small piece of zinc oxide tape. This same needle or another needle may then be used in the pulmonary artery or the left atrium for whatever purpose the investigator may desire. Pressures may be measured, samples may be taken, or temperature measuring devices may be placed within these vascular structures.

After completion of an experimental study, the needles are removed and the stilettes are replaced. In the event steel cannulae have been used, the "closing" end pieces are replaced. The wounds are liberally powdered with sulfonamide, dressed with gauze, and covered with plaster of Paris bandage. The bandage must cover the thorax of the animal and the base of the neck in order to adequately protect the wounds and limit infection. A second experimental study can be carried out 24 to 48 hours after the first. By following this procedure, it has been possible to use some animals as many as seven times within a period of 14 days.

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Distribution of Desoxyribose Nucleic Acid in Tumor Nuclei.* (20103)

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The relative amount of desoxyribose nucleic acid (DNA) in somatic diploid nuclei of non-dividing tissues has been shown to be a constant value. Nuclei with geometric multiples (1:2:4) of this value occur in some tissues (1,2). Since adult mouse and rat liver are known to be polyploid tissues(3,4), the geometric progression of DNA amounts in these tissues is probably a reflection of chromosome content(5). In several dividing tissues it has been shown that values occur intermediate between the diploid and twice the diploid amount of DNA. These values have been associated with the observation that in these tissues DNA synthesis occurs sometime in interphase preceding visible prophase. The mitotic index, and the rate and time of DNA synthesis presumably determine the number of such intermediate values(6). Tumor nuclei have been reported to contain increased amounts of DNA per nucleus as measured by both biochemical and absorption spectrophotometric methods(7,8). Chromosome counts of tumors have revealed polyploidy and aneuploidy to be present(3,9-11). It has been suggested that the increase in DNA content of tumor tissues and tumor nuclei is associated with a vaguely defined disturbance in "nucleic acid balance"(7,12). Recent reports have tended to refute this(5,13,14).

Average amounts of DNA, as determined biochemically, do not have the advantages offered by measurements of DNA in individual nuclei for interpreting growth or cell division. This is particularly the case in tissues where polyploidy is common. In the present study, the DNA content of individual nuclei of tumors and homologous normal tissues has been measured in Feulgen-stained sections.

Methods. The DNA content of nuclei of 5 tumors is presented. 1) A methylcholanthrene-induced squamous cell carcinoma in glandular epithelium of the left ventral prostate of the rat(15) is compared with normal prostate epithelium and precancerous squamous cell metaplasia. 2) The Cloudman S-91 melanoma carried in the dba mouse(16) and a benign skin papilloma of spontaneous origin in a C_{57} mouse are compared with skin of a dba mouse. Neuroepidermal cells were not specifically measured as control for the melanoma. 3) A transplanted mammary carcinoma of spontaneous origin (15091a) in an A strain mouse and a mammary adenocarcinoma which arose spontaneously in a dba mouse are compared with normal dba mammary gland. All tissues were fixed in 10% neutral formalin and washed for 24 hours in running tap water. Embedded tissues were sectioned at 15 μ. Relative amounts of DNA in individual nuclei of tumor and control tissues were measured by absorption microspectrophotometry as described by Swift(1) with light of 560 mμ wavelength from a Beckman monochromator. Tissues were stained in Feulgen reagent for one hour after 14 minutes hydrolysis in N HCl at 60°C. Whenever possible tissues to be compared were simultaneously fixed and embedded, and mounted on the same slide for staining. Slight differences between tissues in the distribution of diploid values are probably caused in part by variations in the preparation of tissues.

Results and discussion. The results can be characterized by 3 generalized observations. 1) the interphase nuclei of the tumor tissues examined contained relative DNA amounts which were roughly distributed into classes representing geometric multiples of the lowest class or diploid value (Class I). 2) All tumor tissues examined, with the exception of the benign skin papilloma, showed an increase in DNA amounts as compared with homologous control tissue, and 3) in most of the tumor tissues examined there was a striking occur-

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rence of intermediate values and/or trailing values following the highest DNA class.

DNA classes in multiples of the diploid value have been shown to occur in normal liver (Fig. 1), pancreas, and several other tissues(1,2). Chromosome counts of regenerating liver indicate that the distribution of DNA classes in this tissue is a reflection of the polyploid content of the nuclei(4). Chromosome counts and absorption photometric measurements on some ascites tumors have also revealed a parallelism between DNA content and polyploidy(8-10). The increase in DNA classes—tetraploid (Class II) and octaploid (Class III)—in the tumors measured is thus presumably the result of induced polyploidy in these tissues. Fig. 1, 2, and 3 show the increase in DNA amounts for the rat squamous cell carcinoma, the 2 mammary carcinomas, and the melanoma. The benign epithelial papilloma and the mammary adenocarcinoma show no increase in DNA classes since normal dba skin epithelium and mammary gland contain a tetraploid class. Tumors with unchanged DNA content have been reported(6,7).

How does polyploidy arise in these tumor tissues? Several mechanisms for the production of polyploidy have been described. 1) Beams and King(17) have observed in regenerating rat liver that many polyploid cells originate by nuclear fusion in binucleate cells during mitosis. Chromosomes of 2 diploid nuclei of such cells may be aligned on a single metaphase plate, thus giving rise to tetraploid nuclei. Polyploid cells may also be considered as arising by 2) endomitosis in which chromosome duplication occurs without a breakdown of the nuclear membrane, resulting in a nucleus with a doubled chromosome number, 3) mitotic anomalies in which there is nondisjunction of chromosomes at metaphase or anaphase through either "sticky" chromosomes or spindle abnormalities, and 4) the fusion of interphase nuclei as observed in the livers of thioacetamide injected rats(18). It

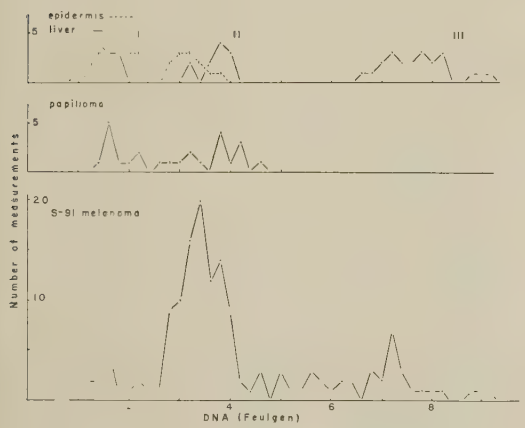

FIG. 1. Amounts of DNA in individual nuclei as determined photometrically on Feulgen-stained sections. Each unit is approximately equivalent to 3×10^{-9} mg DNA. Top: Nuclei from normal C_{57} strain mouse liver and dba strain epidermis. Middle: nuclei from a spontaneous benign skin papilloma in a C_{57} mouse. Bottom: nuclei from Cloudman S-91 melanoma in a dba mouse.

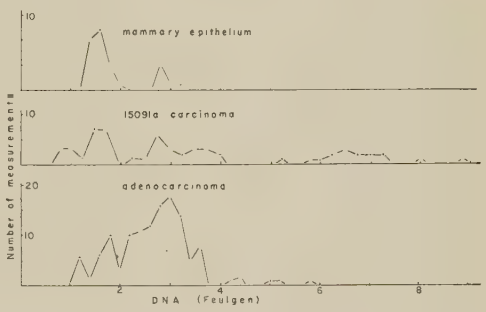

FIG. 2. Amounts of DNA in individual nuclei as determined photometrically on Feulgen-stained sections. Top: normal mammary epithelium from a lactating dba strain mouse. Middle: nuclei from a transplanted mammary carcinoma (15091a) in an A strain mouse. Bottom: nuclei from a spontaneous mammary adenocarcinoma in a dba strain mouse.

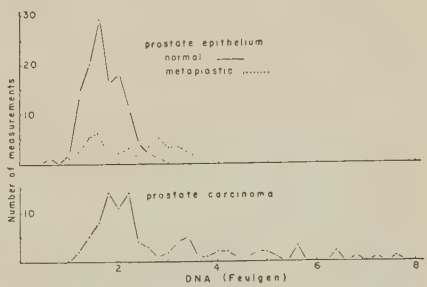

FIG. 3. Amounts of DNA in individual nuclei as determined photometrically on Feulgen-stained sections. Solid lines, top: nuclei from normal prostate epithelium (right side) of a male Sprague-Dawley rat. Bottom: nuclei of a methylcholanthrene-induced squamous cell carcinoma from the same animal (left side). Dotted lines: nuclei of a methylcholanthrene-induced squamous cell metaplasia of a male Sprague-Dawley rat.

is generally apparent that in the tumors studied the formation of polyploidy is concomitant with cell division, and no evidence of endomitosis has been obtained. In some cases, such as in the Cloudman melanoma, Class II nuclei are numerous and actively dividing, and thus seem largely the product of normal cell division.

Intermediate or trailing values in non-dividing mammalian tissues have been rarely encountered(1). Their increased occurrence in the interphase nuclei of dividing tissues and in the tumors examined are presumably largely due to DNA synthesis in preparation for division. Many mitotic figures were observed in the melanoma. In this tissue the occurrence of intermediate values is very striking. Chromosomal abnormalities described in tumor nuclei, such as stickiness, non-disjunction and displacement of chromosomes may also be responsible for some of the intermediate values in these nuclei(11). The differences observed in tumor tissues with respect to the presence and frequency of intermediate and trailing values may be a reflection of rate of growth. The trailing and intermediate values observed in the control prostate and metaplastic prostate epithelium are probably the result of mitotic activity.

The DNA content of some normal tissues of tumor bearing animals, as measured by chemical extraction methods, has been reported to increase(19). Studies in progress, of the DNA content of such tissues on a per nucleus basis as measured in Feulgen-stained sections, reveal that such changes may be due to polyploidy and/or cell number increases (20).

From the data presented here the distribution of DNA values is different in 4 of the 5 tumors from that of the normal controls. It should be emphasized, however, that the tumor values obtained closely resemble those from a number of normal growing tissues where mitosis and polyploidy are present, such as liver in young mice(1). The changes found thus are those to be expected from increased mitosis and polyploidy alone, and do not justify any hypothesis of nucleic acid disturbance(7) or "quantitative change in nucleic acid synthesis"(11), as specifically characteristic of tumor cells. Some of the intermediate DNA values found may be associated with aneuploidy rather than DNA synthesis, although small variation in chromosome number also occurs in normal tissues(21). In the control tissues, variations in DNA values about the class mean are undoubtedly caused to some extent by inaccuracies in the measuring technic(6) and possibly also to aneuploidy, although these variables have not been analyzed in the present case. Data from the tumor tissues are in general agreement with the hypothesis of DNA constancy per chromosome set(1,2,22). The changes in DNA of tumors seem best interpreted as only one of a number of manifestations of a basic alteration in cell processes. From the present evidence DNA change cannot therefore in itself be considered a causative factor in tumor formation.

Summary. The relative amounts of DNA per nucleus in individual nuclei of 5 different tumor tissues, measured by absorption microspectrophotometry in Feulgen-stained sections, are largely distributed into classes in a geometric progression (1:2:4). The tumors showed increases in DNA amounts as compared with homologous control tissue with the exception of a benign papilloma. The occurrence of intermediate values and increased DNA classes is considered to be an expression of changes in chromosome content associated with mitosis and polyploidy rather than a disturbance in nucleic acid synthesis.

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Treatment of Nephrotic Syndrome with Interrupted ACTH or Oral Cortisone Therapy.* (20104)

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In previous investigations(1,2) we have demonstrated that serum complement levels are persistently low in all cases of acute and subacute glomerulonephritis. We have also shown that in almost all cases with the nephrotic syndrome complement is low during the edematous phase, and that 24-48 hours prior to the onset of diuresis, serum complement rises to or toward normal. Conversely, 1 to 2 days prior to clinical relapses, complement falls below normal levels(3). This behavior of complement has been considered to be the result of a complement-binding antigen-antibody reaction *in vivo,* wherein the altered kidney and probably, more specifically, the altered basement membrane of the glomerulus, represents the antigen which provokes antibody formation in the body(1,2,3). The low serum complement levels in these diseases are apparently in no way related to urinary excretion of complement. In the nephrotic syndrome we were unable to find complement in the urine. Furthermore, the low complement levels are not related to the excretion of protein, for in spontaneous or ACTH induced remissions the degree of albuminuria often does not change significantly while complement levels rise sharply *prior* to the remission. No correlation between proteinuria and serum complement levels was demonstrated in four cases of Kimmelstiel-Wilson's Disease in which massive proteinuria and normal complement levels were found. In our determinations of serum complement, the technic of which has been reported previously(1,2), 166 normal subjects or patients with diseases other than glomerulonephritis or the nephrotic syndrome have placed the normal level of serum complement between one and 3 units. (Average, 1.78 units, standard deviation 0.587,† Fig. 1).

Complement unit. Complement was freshly prepared from commercial lyophilized guinea pig serum, diluted in steps of 5 from 1:5 to 1:50 and titration was carried out in the same manner as previously described(1) for the unknown serum. Guinea pig serum 0.1 cc was mixed with 0.1 cc inactivated human serum because guinea pig serum is known to have approximately twice the complementary activity of human serum. The dilution of

* This work was in part supported by a grant of the Council on Pharmacy and Chemistry of the A.M.A.

† As the actual shape of the statistical distribution is unknown and the data are not homogeneous enough and numerous enough to determine this shape, a normal distribution function has been assumed for the sake of expedience. While comparisons between the means are not significantly affected by the shape of the distribution functions, no extrapolation beyond the range of $\pm$ one standard deviation appears permissible.

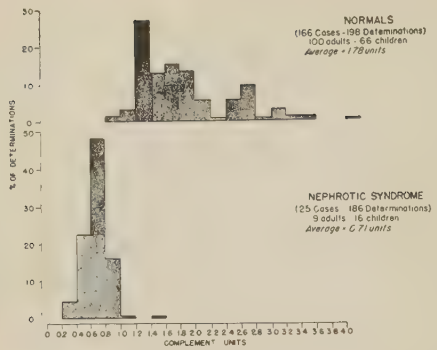

FIG. 1. Distribution of average serum complement levels in 166 normals and 25 patients with the nephrotic syndrome.

guinea pig C′ which just produced 50% hemolysis was defined as a unit of complement (usually a 1:25 dilution). The number of units in the unknown serum was expressed as a multiple of this standard. Thus if the serum under investigation produced 50% hemolysis in a 1:5 dilution its complement content was $5/25 = 0.2$ units.

If the assumption is true that the complement binding antigen-antibody reaction causes a lowering of complement in the host, then depression of antibody formation should effect a rise in serum complement level. Numerous investigators have shown that ACTH and Cortisone depress antibody formation. The degree of depression depends upon the amount and strength of the antigen and the species of the host(4-7). Administration of ACTH and Cortisone does not alter the serum complement level in normal experimental animals or normal humans(8,2).

Procedure. For seven consecutive days ACTH (100 mg per day‡) was given to 16 cases (4 adults, 12 children) with the nephrotic syndrome. No differentiation was made between those patients showing and those not showing signs of underlying glomerulonephritis, for these cases apparently do not differ immunologically. All had low serum complement levels before treatment. (Average 0.71 unit, standard deviation 0.239) Fig. 1. Altogether 25 courses of ACTH therapy were given. Massive diuresis occurred following 22 of the 25 courses (88%). Each diuresis was preceded by a distinct rise in complement and in some cases there was a "rebound phenomenon" characterized by rise in complement to a very high normal level. In the 3 instances where no diuresis occurred complement rose not at all or only very slightly and never approached the normal range. Of the sixteen cases thus treated several relapsed within a few days after diuresis and a total of 11 (69%) relapsed with an associated drop in complement within a period of 12 months.

It has been suggested that after the cessation of ACTH therapy, antibodies are formed at pretreatment rate(6) and clinical relapses occur. On this basis, immediately following diuresis 2 of our cases were given continuous "maintenance" therapy of 25 mg of ACTH daily. In both cases relapse, preceded by lowering of complement, occurred while the patient was on "maintenance" therapy because this dosage is apparently insufficient to depress antibody formation satisfactorily. To obviate untoward effects of sustained high levels of ACTH or Cortisone therapy, but at the same time to obtain sufficient depression of antibody formation as documented by normal complement levels, the following interrupted treatment schedule was devised: Six children with the nephrotic syndrome were treated with a course of 100 mg of ACTH daily for 7 days (25 mg q 6 h.) Diuresis occurred in all cases between the 9th and 12th days. Five to 7 days after ACTH therapy, the following interrupted schedule of treatment was instituted: 100

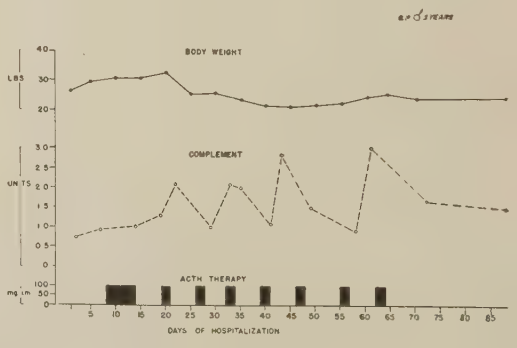

FIG. 2. Complement and body weight in a child with the nephrotic syndrome under ACTH treatment followed by 7 maintenance courses.

‡ We wish to thank Drs. A. H. Holland and G. W. Bissell of the Armour Laboratories for supplying us with ACTH.

mg ACTH daily for 3 consecutive days to be repeated weekly on the same days of the week for 5 to 8 weeks (Fig. 2). Five patients thus treated have not relapsed to date (6-26 mos. after therapy). One child reaccumulated edema 4 days after the onset of a severe purulent otitis media, but this edema promptly disappeared after one additional 7 day course of ACTH and has not reappeared. In all patients, serum complement has remained within the normal range. Three patients have lost all laboratory evidence of the disease (albuminuria, hypoproteinemia and hyperlipemia) while the others still show chemical alterations in urine and blood.

During the past 4 months, we have treated 3 patients with the nephrotic syndrome with ACTH and then interrupted Cortisone therapy. The initial week of ACTH therapy (100 mg per day) was given, and diuresis preceded by rise of complement to normal occurred on the 9th, 10th and 11th days, respectively. 5 to 7 days after ACTH therapy, oral Cortisone§ (100 mg q.i.d.) was given for 3 successive days and then repeated weekly on the same days of the week for 5 additional weeks in one case and 6 additional weeks in the other two cases until serum complement was stabilized at average normal level or higher (Fig. 2). All cases are free of edema and serum complement has remained normal. The urine of one patient is free of abnormal constituents, the other two show a marked decrease in albuminuria. All 3 patients have returned to normal cholesterol levels and the blood proteins are well within the range of normal with a return of the previously inverted A/G ratio to normal. These changes occurred within 4 weeks from the start of Cortisone therapy.

§ We wish to thank Dr. S. Fromer and Dr. E. Alpert of Merck & Co. for supplying us with liberal amounts of Cortisone.

It is too early to predict that this type of interrupted treatment will permanently relieve the nephrotic syndrome. We are reporting our results, however, so that other groups may evaluate an apparently promising therapeutic regime based on the effect of ACTH and Cortisone on the underlying immunologic process.

Summary. 1. Lowering of serum complement during the edematous phase of the nephrotic syndrome is apparently the result of an antigen-antibody reaction. 24 to 48 hours preceding spontaneous or ACTH induced diuresis, serum complement rises significantly to or toward normal. 2. ACTH, given for 7 days, effects clinical remissions which are only temporary in most cases. When the initial week of ACTH is followed by interrupted courses of ACTH given on 3 successive days of each week for 5 to 8 weeks, remissions have been long-lasting. Courses of interrupted therapy were continued until the complement level stabilized at average normal or high levels. Recent work suggests that if the initial treatment with ACTH is followed by interrupted courses of oral Cortisone, similar favorable results may be obtained.

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Water, Sodium, and Potassium Content of Human, Guinea Pig, and Rabbit Lung.* (20105)

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The electrolyte content of pulmonary tissue has met with but limited interest, as is indicated by the scarcity of data found in the literature(1-5). This is remarkable in view of the variety of conditions where large amounts of fluid accumulate in the lung. Such aggregations of fluid, under the clinical term "pulmonary edema," have been extensively investigated as to their pathogenesis, and their experimental production has been recently reviewed by Gamble and Patton(6). Hemingway(7) has contributed nitrogen values for guinea pig lungs.

The experiments reported here were undertaken to establish suitable methods for the determination of water and electrolyte content of lung and vascular tissues as a background for further investigation.

Material and methods. The tissues under examination were lung, blood, and plasma of the guinea pig, rabbit, and man. The guinea pigs were of an average weight of 250 g. The rabbits averaged 4 kg body weight. The diet of the guinea pigs consisted of cabbage, salt, and oats *ad lib*. The rabbits received water in addition to the guinea pig diet. Two agents were used for *anesthesia*: ether to the point of unconsciousness to prepare for heart blood sampling, followed by ether until death occurred; sodium pentobarbital (Nembutal), 0.2 g/kg for cardiac puncture and 0.4 g/kg for sacrificing. In all instances the time that elapsed from initial unconsciousness to removal of the lungs did not exceed 15 minutes. This period was kept to a minimum to avoid lung edema formation(9). The guinea pig lung and blood specimens were pooled in sets of 2 to furnish sufficient material for analysis. Blood intended for *plasma electrolyte* studies was collected under oil with a minimum amount of heparin as anticoagulant. Centrifugation followed by separation of the plasma from the cells was performed immediately. Studies on *human tissue* were done on lungs removed surgically for carcinoma (Specimens A and C) or bronchiectasis (B). The specimen employed for analysis was taken distant from the lesion. Histologic control revealed for lung C an occasional small group of macrophages with pigment in their cytoplasm that stained positively for iron. Specimens A and B were free of lesions. *Gravimetric* sampling was used throughout. For the measurement of water content of lung, blood, and plasma, 3 different methods were used: (a) Oven drying to constant weight. This consisted, after experimentation to determine the proper drying period, of heating the sample overnight (16 hours) in an electric oven maintained at $97 \pm 1°C$. (b) Freeze drying for the same length of time. The vacuum attained was sufficient to maintain the samples in the frozen state until dry. (c) The Karl Fischer titrimetric method(8). Sodium acetate $3H_2O$, reagent grade, as suggested by Warren(10), was employed as the primary standard for water and yielded almost identical duplicates. The liquid specimens were weighed according to Cook *et al.*(8). The lung samples were weighed on small glass spoons suspended from the hook of the balance. The spoon plus sample was then placed in a dry 50 ml volumetric flask. Use of the glass spoon eliminated titration of a paper blank. Karl Fischer reagent was added to within about 10 to 20% of the previously estimated end point. The flasks were stoppered and allowed to stand with occasional shaking for one-half hour before completing the titration. Evidence that the one-half hour extraction period was sufficient consisted of allowing the completely titrated specimen to stand sealed overnight. Such treatment did not result in fading of the end point. *Sodium and potassium* were determined by means of a Beckman DU

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TABLE I. Comparison of Drying Methods.

Species	Oven drying % water			Chemical % water			Freeze drying % water		
	Lung	Blood	Plasma	Lung	Blood	Plasma	Lung	Blood	Plasma
Rabbit 101	80.9	86.5	92.7	81.0	84.6	91.1	80.7	85.9	92.6
102	78.6	84.8	91.5	78.9	84.3	91.5	78.9	84.8	91.3
110				80.9	83.5	90.5			
Human A	80.2	78.5	91.1			89.8	79.9	78.2	91.0
T		78.3	90.2			91.4		78.4	90.2
H		81.0	90.7					81.1	90.5
Guinea pig set 1-10*							79.7±.41	82.6±.43	
11-15†							80.2±.35	82.5±.15	93.5±.2
Rabbit 1‡	81.2	83.0	92.8						
2‡	79.2	81.9	90.6						

* Ether anesthesia. † Nembutal anesthesia. ‡ Data of Manery and Hastings.

Spectrophotometer with flame attachment utilizing acetylene and oxygen. The tissues were ashed in an electric oven at 400°C for 72 to 120 hours, cooled, taken up in 1 ml conc. HCl, and diluted to an appropriate volume for analysis. *Chloride* was measured by the method of Van Slyke and Sendroy(11). To eliminate any possible adsorption by protein, a modification of Hill's method(12) was developed for the estimation of iron. An accurately weighed sample of approximately 100 mg of dry, or a corresponding amount of wet, material was placed in a 50 ml Erlenmeyer flask. Concentrated nitric acid in 0.2 ml portions was added, followed by gentle heating over a free flame to dryness between each addition of acid. A char was formed which was later consumed upon further nitric acid digestion. After oxidation of the char, 1 ml of concentrated HCl and 2 ml water were added to the flask, which was then covered with a watch glass and heated gently on an electric hot plate for about 15 minutes. Upon cooling, the solution was rinsed with water quantitatively into a 10 ml volumetric flask and diluted to the mark with water. One ml of this solution was used for analysis. One ml of a solution containing 20 γ iron per ml (as ferrous ammonium sulfate) was employed as the standard, and one ml of water as the blank. To the test tubes containing these one ml amounts were added in turn: 2 ml water; 5 ml ethyl alcohol; 1 ml 0.2% a-a′ dipyridyl; and 1 ml 2% freshly prepared aqueous iron-free sodium hydrosulfite(12). The tubes were mixed by inversion and allowed to stand for 2 hours for complete color development before reading in a Coleman Junior Photometer at 520 mμ against the blank. An occasional nitric acid blank was run. Recovery of iron was checked by adding 100 μg quantities of iron to duplicate samples of tissue. Recoveries ranged from 99.4% to 101.4%. The optical density versus concentration curve, which passed through the origin, was linear to an optical density of 0.4. At D = 0.8 the drop in the curve was such as to yield values about 7% low. In addition, a simple extraction procedure for measurement of *hemoglobin* in lung tissue was developed and used for human lungs B and C (to be published). The results are comparable to those obtained using the iron determination method.

Results and discussion. Table I lists the water content in percent of total weight of the respective specimens. The differences between the results obtained by the 3 methods are minimal and of the same order as those between individual samples. They also compare well to those reported by other authors by oven drying and by the Karl Fischer method (8). By virtue of its simplicity and speed, the titrimetric procedure of Karl Fischer seems to be the method of choice for determination of water content.

The fact that values for water obtained on freeze dried guinea pig specimens after ether or nembutal anesthesia are nearly identical indicates that, under the experimental conditions, the influence of these 2 types of anesthesia on guinea pig lung water, if any, is the same.

TABLE II. Chloride Data Obtained from Fresh, Oven Dried, and Freeze Dried Specimens.

Material		Chloride, mEq./kg Fresh	Oven dry	Freeze dry
Rabbit 101	Lung	49.4	51.3	50.7
	Blood	100.5	86.7	97.9
	Plasma	99.7	97.1	94.3
Rabbit 102	Lung	63.8	59.6	61.1
	Blood	90.5	88.7	89.2
	Plasma	101.2	97.4	98.8
Human A	Lung	72.6	72.9	
A	Blood		73.7	73.1
A	Plasma	102.8	95.5	94.1
T	Blood	79.2	82.0	73.0
T	Plasma	102.2	102.6	95.3
H	Blood	83.1	71.1	
H	Plasma	92.0	85.7	88.1
R	Plasma	88.7		85.8

TABLE III. Iron Values Obtained from Fresh, Oven Dried, and Freeze Dried Specimens.

Material		Iron, mg/kg Fresh	Oven dry	Freeze dry
Rabbit* 101	Lung	67.1	73.1	72.6
	Blood	211.0	193.0	207.0
Rabbit† 102	Lung	80.1	83.3	80.3
	Blood	241.0	236.0	249.0
Human A	Lung	236.0	234.0	242.0
A	Blood		505.0	515.0

* Hematocrit 15. † Hematocrit 31.

TABLE IV. Sodium and Potassium Content of Oven Dried and Freeze Dried Samples.

Material		mEq. sodium per kg wet material: Oven dried	Freeze dried	mEq. potassium/kg wet material: Oven dried	Freeze dried
Rabbit 101	Lung	76.0	74.3	57.0	61.0
	Blood	112.0	112.0	24.2	24.5
	Plasma	145.0	136.0	3.7	3.7
Rabbit 102	Lung	45.6	43.3	36.6	33.0
	Blood	74.9	81.1	19.9	20.5
	Plasma	122.0	134.0	2.3	2.7
Human A	Lung	86.2	83.7	51.7	51.8
	Blood	63.7	64.5	53.4	54.1
	Plasma	131.0	130.0		

The chloride values obtained on rabbit and human samples are reported in Table II. Those of the dried specimens were calculated to the wet basis, using the percentage of water found by the respective drying method. The results show almost uniformly lower chloride values for the oven and freeze dried samples. This may be attributed to loss of chloride by volatilization, or an absorption of water by the dried materials during storage after the original weighings to determine water content. The latter explanation will be considered in reference to the data presented in Table III.

The values for iron (Table III) were calculated in the same way as those for Table II, and were close for oven, freeze dried, and fresh samples. This indicates that there was no absorption of moisture during storage or weighing. Thus, volatilization of chloride during drying apparently accounts for the loss of this ion.

Table IV reports sodium and potassium values obtained on oven dried and freeze dried samples which were ashed at 400°C for 72 hours. If either drying method were superior for sodium or potassium from the standpoint of volatility, the ashing eliminates the advantage. The values show variation up to (mean) $\pm 5\%$.

The data applied to lung tissue are summarized in Table V. The electrolyte values for blood-free wet tissue were calculated on the assumption that non-vascular lung iron was negligible and that lung blood has the same iron, water, and electrolyte composition as heart blood. These assumptions permitted estimation of the amount of blood, and its corresponding water and electrolytes, present per unit weight of lung. After subtraction from the total, the remaining water and electrolytes were those of the lung *per se*. Rat and rabbit lung data of Manery and Hastings (5) are included for comparison.

A relatively small range of values for the guinea pig lung electrolytes will be noted. Darrow *et al.*(13) have published values for electrolytes of muscle, heart, and liver of the cat, and Harrison's group(14), data on muscle and liver of the dog, monkey, and rabbit. Their values likewise show small variation of electrolyte content of any of the 3 tissues of the species examined, although there was considerable variation from one species to another. The electrolytes of the rabbit lung (Table V) are, on the other hand, quite variable. In this connection, it should be noted that the rabbits used in these experiments were severely anemic. Also, in com-

TABLE V. Na^+, Cl^-, and K^+ in Milliequivalents per kg of Lung Tissue.

Tissue	Na^+	Cl^-	K^+	Na^+	Cl^-	K^+	Na^+	Cl^-	K^+
	Per kg wet lung			Per kg blood free wet lung			Per kg blood free lung water		
VAHO*									
Avg G. pigs 1-10	94.1 ± 3.5		85.3 ± 4.6	88.9 ± 6.5		93.7 ± 6.1	113.2 ± 8.0		113.8 ± 15.1
11-15	92.5 ± 2.7	46.7 ± 3.6	84.1 ± 1.7	87.6 ± 4.3	36.5 ± 5.5	90.4 ± 2.6	110.8 ± 7.5	46.5 ± 7.2	105.6 ± 2.2
Human A	85.0	72.6	51.8	82.2	64.8	37.2	95.0	74.9	57.0
B	76.8	74.3	48.1	72.4	73.8	52.9	91.3	93.1	66.7
C	73.4	62.0	39.2	67.8	62.0	37.5	81.3	67.5	46.0
Human lung, mean and dev.	78.4 ± 4.4	69.6 ± 5.1	46.4 ± 4.8	74.1 ± 5.4	66.9 ± 4.5	42.5 ± 6.9	89.2 ± 4.6	78.5 ± 8.5	58.6 ± 8.2
Rabbit 101	70.7	49.4	62.8	51.9	30.8	83.4	66.7	39.6	107.3
102	44.5	63.8	34.8	21.8	36.7	33.7	27.1	45.6	41.9
110	45.2	46.0	42.8	36.6	36.0	48.2	45.6	44.8	60.0
Manery & Hastings	Per kg wet tissue			Per kg blood and fat free fresh lung			Per kg blood free lung water		
Rat No. 6		65.1			61.6				
No. 7		57.2			51.3				
Avg. 5 rats								71.0	107.2
Rabbit No. 1	72.3	67.3		60.8	57.7				
No. 2	71.5	63.6		62.6	55.4				

* Veterans Administration Hospital, Oakland, Calif.

paring results, a source of variation is to be found in the manner used to correct for blood values. While the foregoing authors mentioned(13,14) utilized exsanguination, probably 40 to 50% complete, the present data are based on iron determinations.

If all chloride is considered to be extracellular, the extracellular tissue water of guinea pig lungs (Fig. 1) is about 30% of the total blood-free lung water. The corresponding value, calculated from Manery and Hastings' data on rats, is 40%. The 3 rabbits vary from 27 to 52%. The calculated extracellular water of human lungs was 56.8, 58.3, and 59.5% and is, therefore, considerably greater than in guinea pig lungs. This may be accounted for by species variation as well as by the ligation of the pulmonary vein prior to that of the pulmonary artery. The latter is done to prevent escape of tumor cells into the blood leaving the lung. Although the interval between the 2 ligations is short, extravasation of plasma may take place.

Conclusions. 1. A comparison of 3 methods for water determination of lung, blood, and plasma has been given. The method utilizing the Karl Fischer reagent is considered superior. Ratios of iron, sodium, potassium, and chloride content of guinea pig, rabbit, and human lung are presented. 2. The probable volatilization of chloride during oven or freeze drying is discussed. The electrolyte concentrations and the extracellular water vary

FIG. 1.

widely in the lungs of rabbits, less so in guinea pigs, but are not as consistent as has been reported for rats. The extracellular water in the 3 human lung specimens was remarkably constant.

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Use of Trypsin in Preparing Subcultures of Monkey Testicular Tissue.* (20106)

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The use of trypsin as a method of obtaining tissue culture cells for transplanting purposes was introduced by Rous and Jones(1) and elaborated by Vogelaar and Erlichman(2). A detailed study of the stimulating effect of various proteolytic enzymes on tissue growth was reported by Simms and Stillman(3). Several interesting observations were made by these various authors: a) Contact with 3% crude trypsin for 1 hour at 37°C did not injure rodent or avian cells, but 5% did(1); b) Following solution of the plasma clot, repeated subculture was possible(1,2); c) Cell growth was rapid and at times explosive (1,3); d) The lag period for adult cells in tissue culture was shortened(3); e) Similar stimulation was obtained with both crude and crystalline trypsin, chymo-trypsin, and papain, suggesting that proteolysis of growth inhibitory substances was an essential factor (3). The purpose of this report is to present a simple tryptic digest method of transplanting roller tube cultures of monkey testicle with the objective of increasing the yield of usable explants for the propagation of poliomyelitis and other viruses.

Methods. Propagation of original tissue. Freshly obtained rhesus monkey testicles were minced in Simms-Hanks solution (1 part ox serum ultrafiltrate and 3 parts Hanks' balanced salt). Six to 8 fragments per tube were planted in chicken plasma which was then clotted with 50% chick embryo extract. The nutrient fluid consisted of 1.5 ml of a medium containing 10% chick embryo extract (1:1), 78.75% Simms-Hanks solution, 10% unheated horse serum, and 1.25% human albumin. Penicillin and streptomycin were added in a final concentration of 100 units and 100 μg per ml, respectively. The tubes were placed in the roller drum at 37°C and incubated until well developed fibroblast outgrowth was obtained (2 weeks). The cells were maintained thereafter by adding fresh medium at weekly intervals.

Subculture. The nutrient fluid and the original tissue fragments were removed leaving the outgrowth intact. To each tube was

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added 1 ml of 0.5% trypsin in Ringer's solution that had been freshly prepared from a filtered 2% crude trypsin stock(2,3). The culture tubes containing trypsin were returned to the roller drum and incubated for 1 hour at 37°C, at which time dissolution of the clot was usually complete. Simms-Hanks mixture (10-12 ml) was then added and the tubes were rotated in an angle centrifuge at 2500 rpm for 5 minutes. The supernate was discarded and the cells were washed once again in the same manner and recentrifuged. The sediment was suspended in 1.5 ml of 50% chick embryo extract and the cell clumps dispersed by gentle suction back and forth through a small bore capillary pipette. With the same pipette 2 drops of the cell suspension were spread lengthwise over the inner surface of a tube which had previously been coated with chicken plasma. When clotting was complete, 1.5 ml of nutrient fluid was added and the tubes were placed in the roller drum.

Results. Initial proliferation of fibroblasts was unusually vigorous and distinct growth originating from small clumps and single cells was observed in 24 hours. By 6 to 7 days all the tubes containing transplants were suitable for virus inoculation. The yield from one tube of original culture averaged 15 subcultures, which required an estimated working time of approximately 30 minutes for the entire procedure. A stock of primary cultures may be prepared when the testicle is first obtained and at selected intervals subcultures can be made thus insuring a continuous supply of young fibroblasts over a period of a month or longer. In a few experiments with human tissue, successful subcultures have been propagated from material kept in the roller drum for as long as 3 months.

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Effect of Hyaluronidase on Inflammation.* (20107)

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Investigations with hyaluronidase have shown that the spreading properties of the enzyme permit more effective application of certain drugs(1). The effect of this enzyme on local inflammation due to irritating drugs, however, was not known; and these experiments were designed to study this aspect and to evaluate the applicability of hyaluronidase as an adjuvant in therapy with irritating agents.

Two strong irritants were used: the oil-soluble allylisothiocyanate and the water-soluble paraphenylenediamine. Gross and microscopic observations on animals treated with these irritants showed that in the presence of hyaluronidase, the local inflammatory

TABLE I. Effect of Intradermal Paraphenylenediamine in the Presence and Absence of Hyaluronidase (End of 3 Hr). 6 rabbits per exp.

Treatment*	Reaction area†	
	No. reacting	Avg area of dye ($mm^2 \times 10^2$)
.9% NaCl + .9% NaCl	1	.02
.9% NaCl + H	5	.23
4% P + .9% NaCl	6	11.98
4% P + H	6	21.96

* H = Hyaluronidase. P = Paraphenylenediamine.

† None of the rabbits showed petechiae. Only the rabbits (6) inj. with 4% P + .9% NaCl showed edema.

* Presented at meeting of Fed. of Am. Soc. for Exp. Biol., Section on Pharmacology and Experimental Therapeutics, New York City, Apr. 14-18, 1952.

TABLE II. Effect of Intradermal Allylisothiocyanate in the Presence and Absence of Hyaluronidase (End of 3 Hr).

Treatment*	No. rabbits used	Reaction area: No. reacting	Reaction area: Avg area of dye ($mm^2 \times 10^2$)	Intensity of inflammation: No. showing petechiae	Intensity of inflammation: Avg area of petechiae ($mm^2 \times 10^2$)	Intensity of inflammation: No. showing edema
M + .9% NaCl	13	3	.04	0	0	0
M + H	13	10	.92	0	0	0
2% A in M + .9% NaCl	16	16	21.3	16	2.8	16
2% A in M + H	16	16	39.4	16	4.6	2
.25% A in M + .9% NaCl	13	13	8.1	6	.42	13
.25% A in M + H	13	13	16.8	8	.89	1

* M = Mineral oil. A = Allylisothiocyanate. H = Hyaluronidase.

response was modified.

Method. The trypan blue irritation test procedure described by Menkin(2) was modified and used in this study. This test is based on the observation that intravenously injected colloidal dyes seek out and stain injured skin areas as a result of increased vascular permeability(3-5). Forty-six albino rabbits were used. The restrained animals were injected intradermally on the clipped abdomen with 0.3 ml of either allylisothiocyanate in mineral oil or aqueous paraphenylenediamine. Within 1 minute after the injection of the irritants, 0.1 ml of hyaluronidase (900 turbidity reducing units†) was introduced into some of the injection sites; the remaining sites were injected with 0.1 ml of physiological salt solution (Tables I and II). The vehicles with and without hyaluronidase were injected as controls. Fifteen minutes after the intradermal injections were completed, 10 mg/kg of trypan blue was injected into the marginal ear vein of each rabbit and observations on the injection sites were made periodically for 3 hours. The area of spread of extravasated dye at various time intervals after injection was obtained using the formula D x d x Pi/4 (assuming an elliptical shape for the bleb) where D is the longest diameter and d the shortest diameter. Other animals similarly treated were observed and sacrificed at the end of 24, 48, 96 and 192 hours. At sacrifice, the injection sites were excised and prepared for microscopic evaluation. Hematoxylin-eosin stain and Beyer's trichrome stain were used on the sections.

Results. Tables I and II summarize the gross observations on rabbits following intradermal injections of either paraphenylenediamine or allylisothiocyanate in the presence and absence of hyaluronidase. The most striking feature is the absence of swelling (edema) at the sites where the irritants were administered with hyaluronidase; however, the area of extravasation of dye at these sites is approximately 2 times greater. While petechial spots characteristically appear only after injections of allylisothiocyanate, they are more numerous and appear earlier when the injections are supplemented with hyaluronidase. The gross appearance of these lesions is shown in Fig. 1 (A,B).

A number of days after the initial exposure to the irritants the reaction areas are still identifiable grossly; and the differences between the sites that received the irritants with and without hyaluronidase are easily recognized. The lesions resulting from the irritants administered without supplemental hyaluronidase have a firm consistency. Elongated masses limited to the under surface of the skin extend in several directions from the injection sites and are glistening and gelatinous in appearance. The areas exposed to the irritants administered with supplemental hyaluronidase are, in contrast, soft and pliable.

The microscopic appearance of the injection sites at the end of 24 hours is shown in Fig. 2 (A-F). Sections A (physiological salt solution) and D (mineral oil) show the changes due to injection of the vehicles. In general, there is minimal separation of the epidermis, fatty connective tissue and muscle layers. Vacuoles are present in the corium of the mineral oil section only and show the dispersion of the oil droplets.

† Wydase,® Wyeth.

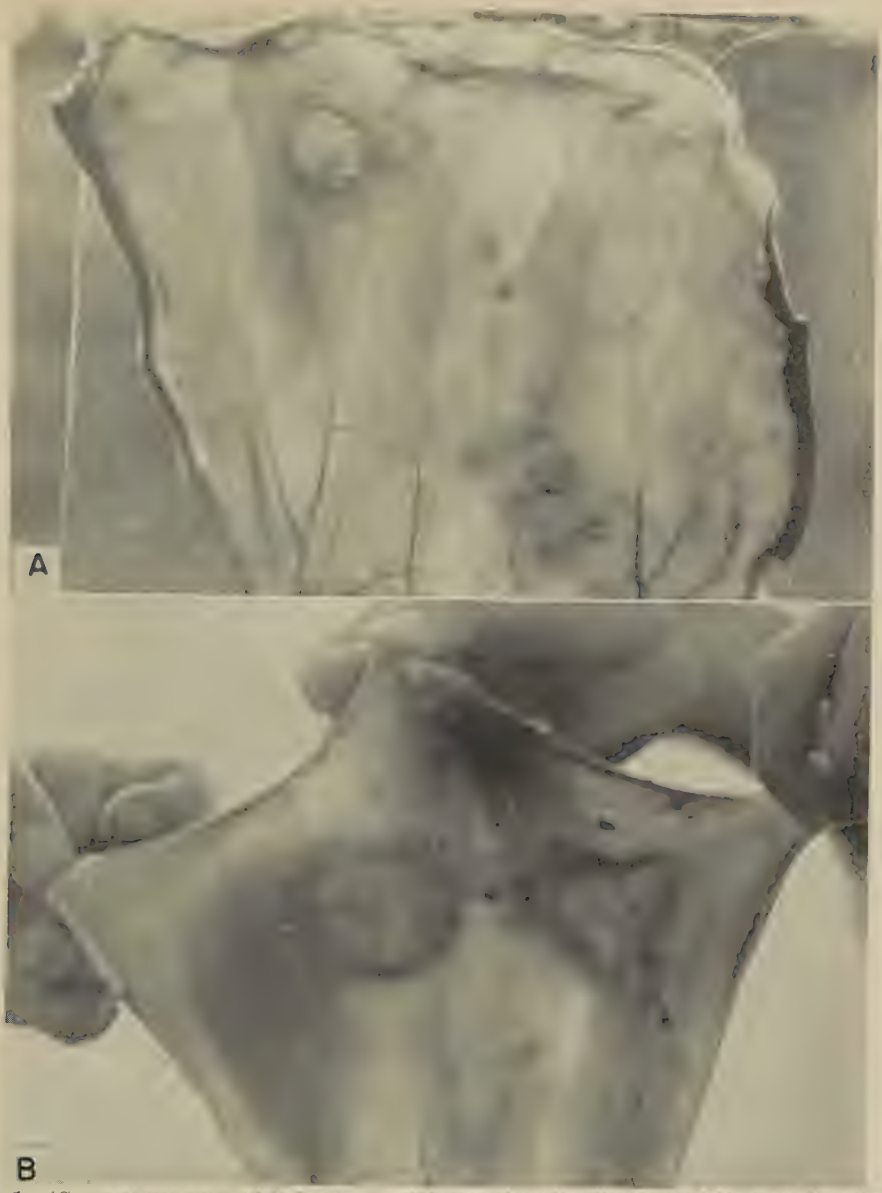

FIG. 1. (Gross appearance of lesions). A: 4% paraphenylenediamine; abdominal skin viewed by transmitted light (end of 24 hr). Upper left, with physiological saline (note extravasation of dye with suggestion of swelling lateral to lesion); lower right, with hyaluronidase. B: 2% allylisothiocyanate in mineral oil; abdominal skin viewed by transmitted light (end of 24 hr). Left, with physiological saline (note the gelatinous thickening of tissue lateral to the lesion with extravasation of dye in this area); right, with hyaluronidase.

The swelling seen grossly at the sites of injection of the aqueous irritant, paraphenylenediamine, or the oil-soluble irritant, allylisothiocyanate, is seen microscopically as distortion of the tissue architecture with separation of the fibers and cellular elements (sections B,E). The oil droplets (vacuoles) conspicuous in the tissue exposed to allylisothiocyanate (section E) further contribute to the over-all distortion. At the same magnification both sections almost completely fill the entire microscopic field. In comparison,

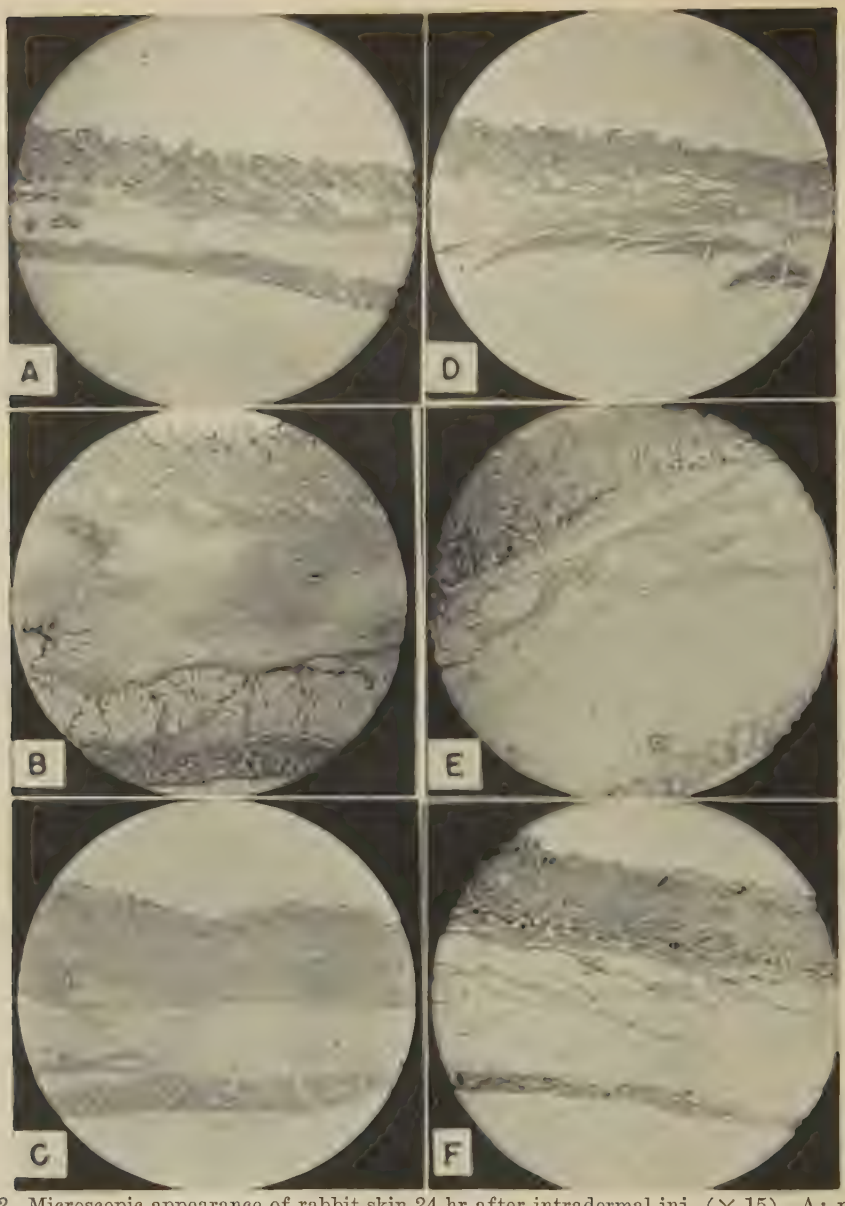

FIG. 2. Microscopic appearance of rabbit skin 24 hr after intradermal inj. (× 15). A: physiological saline; B: 4% paraphenylenediamine + physiological saline; C: 4% paraphenylenediamine + hyaluronidase; D: mineral oil; E: 2% allylisothiocyanate in mineral oil + physiological saline; F: 2% allylisothiocyanate in mineral oil + hyaluronidase.

sections taken after administration of the irritants with supplemental hyaluronidase (sections C,F) are considerably thinner, show less tissue distortion and less separation of fibers. The oil droplets due to the allylisothiocyanate, however, are still conspicuous (section F).

Table III summarizes the chief microscopic findings as they relate to injury and repair of the skin following contact with the irritants for various lengths of time. Here the separation of tissue elements, fibrosis and degree of extravasation of erythrocytes are scored on a 1 to 5 basis. In brief, it can be said that there is no microscopic evidence that the degree of vasodilatation, number of polymorphonu-

TABLE III. Summary of Microscopic Findings* (Avg 6 Rabbits per Group).

Treatment†		Response of tissue (by hr)						
		Separation of elements			Hemorrhage			Fibrosis‡
		3	24	192	3	24	192	192
4% P	+ S	3.5	2.7	.5	1.8	2.5	2.3	3.3
	+ H	2.3	3.0	.6	2.7	2.2	2.5	2.8
2% A in M	+ S	4.5	3.7	1.2	3.3	3.0	2.0	3.0
	+ H	3.0	2.3	.2	3.8	3.5	2.5	2.3

* Scores are on the basis of 1 to 5.

† P = Paraphenylenediamine. A = Allylisothiocyanate. S = Physiological saline. H = Hyaluronidase. M = Mineral oil.

‡ Fibrosis occurred in all animals between 24 and 192 hr.

clear leukocytes, number of lymphoid cells or the number of spindle cells occurring at the irritation sites are affected by the use of hyaluronidase. Fibrosis is scored at 8 days and does not appear to be significantly less where hyaluronidase is used with the irritants. On the other hand, however, where hyaluronidase is used in conjunction with the irritants, sections of these sites taken after 3 hours show more extravasated erythrocytes than those sites where the irritants are administered alone; even after 8 days the sites show evidence of hemorrhage.

Discussion. This problem was undertaken for practical purposes. The local irritating properties of certain drugs sometimes preclude their parenteral use; but under certain conditions the tissue response to the presence of irritants can be altered so that less injury and discomfort results.

The non-toxic mucolytic enzyme hyaluronidase seemed to be the agent of choice for two reasons. Because of its power to hydrolyze the hyaluronic acid barrier found in various tissues of the body, hyaluronidase promotes the diffusion of irritants. Furthermore, the action of hyaluronidase on the structures surrounding and supporting the capillary wall, while not altering the endothelium *per se*(6), results in increased filtration across the affected area. These factors operating together at the site of the injury serve both to lower the local concentration of the irritants and aid in their removal; in this way the damaging effects on the surrounding cells are reduced.

The swelling due to edema and pressure exerted thereby on underlying structures are also eliminated. The edema, which in the absence of supplemental hyaluronidase cannot easily escape or move about, diffuses rapidly away from the site of injury; and this diffusion continues, according to Hechter(7), as long as edema furnishes pressure and volume for hyaluronidase action. Moreover, elimination of the edema pocket results in minimal distortion of the tissue architecture. This may be of some importance in terms of healing and scar tissue formation.

The picture is somewhat altered when the irritant is oil-soluble. The exudate formed at the site of injury forms an emulsion with the oily irritant; and the oil droplets make their way between the structural elements of the tissue to contribute to the distortion of the architecture. Although hyaluronidase facilitates the diffusion of the oily irritant and the exudate, it is questionable whether this is an advantage. Elimination of the edema phase of inflammation by dispersing the oily irritant and the exudate results in a larger area of tissue in intimate contact with the concentrated irritant. And since oil is absorbed at a slow rate, as indicated by the persistence of oil droplets in the connective tissue for 8 or more days, this allows the irritant to act on this greater area during this time.

The phenomenon of hemorrhage seen in the sections represents the vascular damage resulting from the action of the chemical irritants upon the capillary wall. Although hyaluronidase itself does not produce any signs of local tissue injury(1), its use in conjunction with either allylisothiocyanate or paraphenylenediamine hastens the extravasation of erythrocytes from the damaged vessels. Hyaluronidase accomplishes this by softening the material surrounding and supporting the damaged capillary wall thereby permitting minute

vascular ruptures and petechial hemorrhages to occur earlier and more prominently.

Summary. 1. Hyaluronidase modifies the inflammatory response of tissue due to certain oil and water-soluble irritants. 2. Hyaluronidase affects the vascular response. The spread of trypan blue in the tissues representing increased vascular permeability, appears earlier and is greater when the irritants are administered with hyaluronidase; and where the nature of the irritant is such as to produce hemorrhages, the areas of these hemorrhages are also greater and occur earlier. 3. Hyaluronidase does not alter the solubility or absorption of an oil-soluble irritant. 4. Use of hyaluronidase with oil or water-soluble irritants eliminates edema from the classical picture of inflammation. This may have practical applicability where prevention of edema resulting from contact with irritants is important. 5. These studies also indicate that hyaluronidase is a useful tool for investigating the edema aspect of the inflammatory reaction.

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Use of "Granuloma Pouch" Technic in the Study of Antiphlogistic Corticoids.* (20108)

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The "granuloma pouch" technic has been devised as a procedure for the quantitative analysis of factors regulating inflammation and wound healing. Its principle is that by injecting a given amount of air into the loose subcutaneous tissue of the rat, an almost perfectly symmetrical, spherical or ellipsoid air space, of any desired size, can be created. This acts as a mold for the subsequent formation of a granulomatous membrane, into which the internal surface of this cavity can readily be transformed, by injection of some irritant (*e.g.,* croton oil, formalin, kaolin) into the air space. The thickness and structure of the granuloma pouch, as well as the quantity and constitution (cellular and chemical constituents) of the fluid, which gradually replaces the air, can be largely determined at will by the selection of appropriate irritants. The salient features of this technic and its principal applications have been outlined elsewhere(1,2).

In the present communication, we would like to report upon experiments demonstrating the striking changes which occur, in the granuloma pouch, upon topical administration of hydrocortisone acetate.

Experimental observations. In the *1st experiment,* 16 female Sprague-Dawley rats, weighing 128 to 151 g, were subdivided into 2 equal groups. In all the animals of both groups, the dorsal region was depilated with barium sulfide. Immediately afterwards, the 8 rats of one group were treated with hydrocortisone micro-crystals. The suspension was made up by diluting the original preparation of Hydrocortone Merck (which contains 25 mg per ml) with the original suspension fluid (kindly supplied by Merck and Co., Montreal), in the amount required to obtain a concentration of 10 mg per ml. Of this suspension, 0.1 ml (*i.e.,* 1 mg) was then injected in the interscapular region. In order to obtain

* This work was carried out with the aid of the National Research Council of Canada.

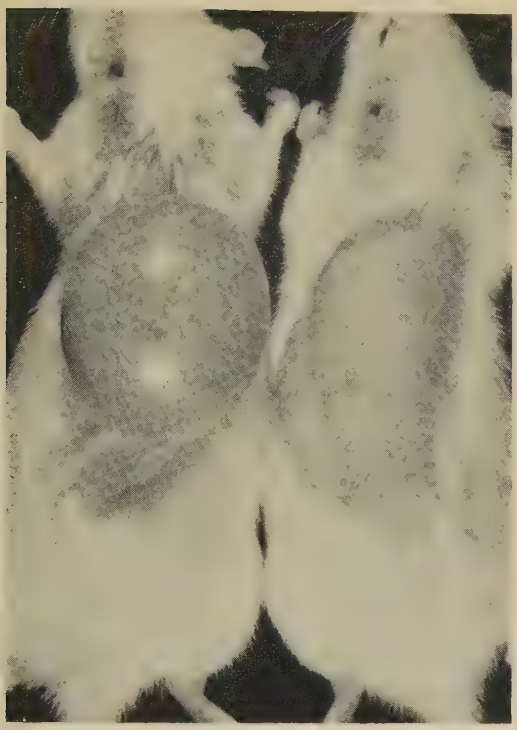

FIG. 1. On left, control rat received no hormone treatment. Note the large "granuloma pouch" filled, under pressure, with hemorrhagic exudate. On right, similarly treated rat in which whole inflammatory response was suppressed by a single topical inj. of 1 mg of hydrocortisone (much of which is visible, near cranial margin of depilated area, in the form of a whitish crystal deposit).

good distribution over a large area, this was done by inserting the entire length of a 3/4 inch, No. 27 hypodermic injection needle just underneath the skin, parallel with the vertebral column, through the loose subcutaneous tissue of the back. By injecting while the needle was then withdrawn, a long streak of hormone deposit was created. The 8 control animals remained untreated. *Twenty-four hours later,* in all animals of both groups, granuloma pouches were made by the following procedure: 25 ml of air were injected deep into the loose connective tissue between the shoulder blades through a No. 27 hypodermic needle. This was immediately followed by the injection of one ml of a 1% croton oil solution (in mazola oil) into the resulting air space, through the same needle. During the *first 2 days,* the air-filled pouches were essentially similar in the 2 groups. However, beginning at about the fourth to sixth day, the wall of the air bubble began to thicken, in the untreated control group, and transillumination revealed that a hemorrhagic brownish fluid was beginning to fill the cavity. In the hydrocortisone treated animals, the walls of the pouch remained so thin, that they could not be palpated and transillumination revealed only the transparent oily solution, which we had originally injected. Throughout the experiment the hydrocortisone deposit remained visible, by naked-eye inspection, just under the skin in the peripheral wall of the pouches. During the following days, the accumulation of exudate and the thickening of the granuloma-pouch wall proceeded constantly in the untreated controls. In the hydrocortisone treated animals, some of the air was absorbed and no fluid was formed to replace it, so that the pouch gradually collapsed.

The experiment was terminated on the 14th day after the croton oil injection, and Fig. 1 illustrates the typical appearance of one rat from each group. In the control animals, the

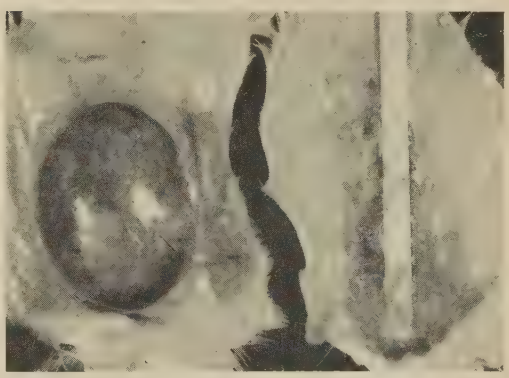

FIG. 2. Another pair of a control (left) and a hydrocortisone treated (right) rat, which underwent same procedure for production of granuloma pouches as in Fig. 1. The dorsal skin was removed, but the pouch remained attached to it, hence, entire region can be viewed from the inside. Again note the thick, non-transparent, granulomatous membrane, which forms a very regular ellipsoid. It is filled, under pressure, with hemorrhagic fluid. The empty pouch itself weighed 3.16 g and contained 40 ml fluid. The pouch of the hydrocortisone treated rat is not delimited from the surrounding tissue. It is so transparent that one can see the millimeter-scale, in its lumen, almost as clearly through the membrane as in those places where it is directly exposed through accidental holes. Perfect dissection was impossible, but even with much adjacent tissue still adherent the dissected mass weighed only 0.40 g.

TABLE I. Effect of Hydrocortisone on Granuloma Pouch (1st Experiment).

Treatment	Controls									Hydrocortisone treated								
No. of rats	1	2	3	4	5	6	7	8	Avg	1	2	3	4	5	6	7	8	Avg
Initial body wt, g	150	133	128	142	146	147	150	145	143	142	138	129	133	151	128	146	134	138
Final body wt, g	165	150	151	152	160	158	161	157	157	161	150	148	149	162	145	160	142	152
Fluid in pouch, ml	12	32	35	28	26	24	25	40	28	*	*	*	*	2.0	*	*	*	—
Air in pouch, ml	3.0	3.1	3.5	3.0	1.0	1.0	3.0	3.2	2.6	3.1	2.1	2.5	2.8	3.0	3.3	3.4	3.2	2.9
Wt of pouch wall, g	3.6	3.8	4.1	4.2	3.1	4.6	3.5	3.1	3.7	0.4	0.3	†	†	2.0	†	†	†	—

* Only about 1 ml of the original oil present; no macroscopically detectable exudate.
† Pouch too thin to be dissected and weighed.

pouch was filled by a large amount of hemorrhagic exudate, which accumulated under considerable pressure and hastened the absorption of air. Consequently, the pouch became extremely hard. On the other hand, in the hydrocortisone treated animals, only a slight elevation of the skin revealed some remnant of the air injected. Here, the skin was quite flabby and the air was obviously not under pressure.

In the control animals, not hormone treated, the pouch could easily be dissected; indeed, after making a skin-incision, mere pressure sufficed for the blunt enucleation of the ellipsoid granuloma pouch. Conversely, in the hormone treated animals, there was no definite membrane to dissect. Upon carefully splitting the skin, some remnant of air and the apparently unchanged oil solution were visible in virtually unchanged connective tissue. In the untreated controls, the pouch contained 1.0 to 3.5 ml of air and between 12 and 40 ml of hemorrhagic exudate. The carefully washed empty granuloma pouch itself weighed between 3.1 and 4.6 g (Fig. 2 and Table I).

Apparently, very small amounts of hydrocortisone suffice to inhibit the inflammatory response under these conditions. It must be kept in mind that with this technic we produced an inflammatory focus whose total weight (granuloma tissue and exudate) amounted to approximately ¼ of the total body weight, yet, as judged by macroscopic estimation, the size of the hydrocortisone deposit under the skin has hardly diminished during the 14 days of this experiment. Obviously, only a very small fraction of the hormone was actually involved in this intense antiphlogistic effect. It is rather important to emphasize, however, that in order to be so effective, the hormone must be injected into the connective tissue, which will subsequently become the granulomatous pouch wall. Numerous control experiments showed that if the same amount of hydrocortisone is injected at a distance of even only 1 mm from the pouch, it is virtually inert.

In a *2nd experiment,* conducted in exactly the same way, we used 3 groups of rats. Group I acted as not hormone treated controls; Group II received 1 mg of hydrocortisone acetate on the first day of the experiment in the same manner as the rats of the first experimental series; Group III was injected with the same amount of hormone directly into the air space. In Group I the granuloma pouch developed as in the untreated controls of the first series; it was completely inhibited in all the animals of Group II, which received hydrocortisone by injections into the adjacent subcutaneous tissue (which subsequently came to form the pouch wall): in the rats of Group III, which were given the same amount of hormone by injection into the pouch cavity, no significant inhibition was noted.

The question arose whether the croton oil solution (which remained in a seemingly unchanged condition within the connective tissue of the air space in the hydrocortisone protected rats) was still irritating to unprotected normal tissues at the end of the 14-day experiment. To examine this, we collected the oil from all the pouches in the hydrocortisone protected group (Group II of the second series) and injected 0.2 ml, just underneath the plantar aponeurosis of the right hind paw, in an additional group of 6 female Sprague-Dawley rats weighing 135 to 146 g. 0.2 ml of mazola oil was injected, as a blank control, into the left hind paw in these same animals.

TABLE II. Comparison between Effect of Hydrocortisone when Injected into Wall or Cavity of Granuloma Pouch (2nd Experiment).

Treatment	Controls									Hydrocortisone in wall									Hydrocortisone in air-space								
No. of rats	1	2	3	4	5	6	7	8	Avg	1	2	3	4	5	6	7	8	Avg	1	2	3	4	5	6	7	8	Avg
Initial body wt, g	120	112	115	130	150	134	150	151	*133*	137	135	125	116	135	140	144	133	*133*	141	131	144	120	134	142	150	140	*138*
Final body wt, g	148	123	145	149	168	142	150	172	*150*	160	142	143	148	150	152	180	153	*153*	160	190	175	145	145	152	160	160	*161*
Fluid in pouch, ml	17	12	19	10	20	27	28	19	*19*	2.6	*	*	*	2.0	*	*	*	—	11.5	32	22	13	12	11	28	17	*18*
Air in pouch, ml	1.0	2.0	1.5	2.0	1.0	0.6	2.0	1.5	*1.4*	1.0	1.5	1.0	2.0	1.5	1.0	1.7	2.0	*1.7*	1.0	0.6	1.2	1.0	2.0	1.5	0	1.5	*1.1*
Wt of pouch wall, g	3.5	3.2	3.6	4.1	4.5	3.5	3.0	3.1	*3.6*	†	†	0.5	0.3	†	†	2.6	†	—	3.0	2.5	1.6	2.5	3.6	2.1	2.0	1.6	*2.4*

* Only about 1 ml of the original oil present; no macroscopically detectable exudate. † Pouch too thin to be dissected and weighed.

TABLE III. Comparison between Effect of DCA and Δ^5-Pregnenolone upon Granuloma Pouch (3rd Experiment).

Treatment	Controls									DCA									Δ^5-pregnenolone								
No. of rats	1	2	3	4	5	6	7	8	Avg	1	2	3	4	5	6	7	8	Avg	1	2	3	4	5	6	7	8	Avg
Initial body wt, g	158	160	141	146	160	159	155	157	*154*	145	140	160	153	160	160	159	161	*155*	158	160	156	140	155	160	146	150	*153*
Final body wt, g	2.01	195	182	183	201	179	182	198	*190*	182	210	194	200	200	195	198	201	*198*	200	204	204	200	220	191	192	198	*201*
Fluid in pouch, ml	20	12	17	19	22	14	17	13	*17*	14	22	14	16	16	20	15	16	*17*	26	10	19	19	15	9	12	*	*16*
Air in pouch, ml	3	4	7	3	5	6	6	5	*5*	5	5	6	5	6	7	5	6	*6*	6	8	7	7	6	5	6	7	*6*
Wt of pouch wall, g	3.6	2.1	3.1	3.6	4.1	2.6	3.0	2.6	*3.0*	3.6	3.4	2.8	3.7	3.4	2.9	3.1	3.4	*3.3*	4.5	2.1	3.8	2.4	2.3	2.6	3.5	3.1	*3.0*

* This specimen was accidentally lost.

FIG. 3. Results of topical-irritation-arthritis tests. On left (top) paw of rat which received 0.2 ml of the oil, recovered from the hydrocortisone-protected "granuloma-pouch" region of the rat illustrated in Fig. 2. Note necrosis, near heel, and some hemorrhagic inflammatory response around ankle region. The other foot (bottom) was injected with same amount of mazola oil for control purposes and shows no reaction. On the right, another animal whose one paw (top) was inj. with 0.2 ml of a fresh 1% croton oil solution and shows a very intense hemorrhagic response throughout. Here again the other paw (bottom) was inj. with ordinary mazola oil and shows no reaction.

For comparative purposes, another group of 6 female Sprague-Dawley rats weighing 134 to 141 g was given 0.2 ml of a fresh 1% croton oil solution in mazalo oil into the right hind paw and again ordinary mazola oil into the left hind paw. Fig. 3 shows the result of this treatment after 24 hours. The mazola oil in itself caused no visible irritation, while the fresh 1% croton oil produced a very marked, hemorrhagic inflammatory response. The oil removed from the pouches of hydrocortisone protected rats was still very irritating, but evidently less so than the croton oil solution which had been originally introduced into the pouch.

It may be concluded that, even after 14 days of sojourn in the pouch, which showed no sign of irritation, the croton oil solution still retained much of its phlogistic effect. It is not possible to judge by our observations whether the decrease in phlogistic potency was due to partial absorption of the croton oil from the mazola oil solvent, to inactivation, or to the admixture of hydrocortisone which may have penetrated into the oil. In any case, however, it is obvious that, under these conditions, the cells of the pouch wall were pro-

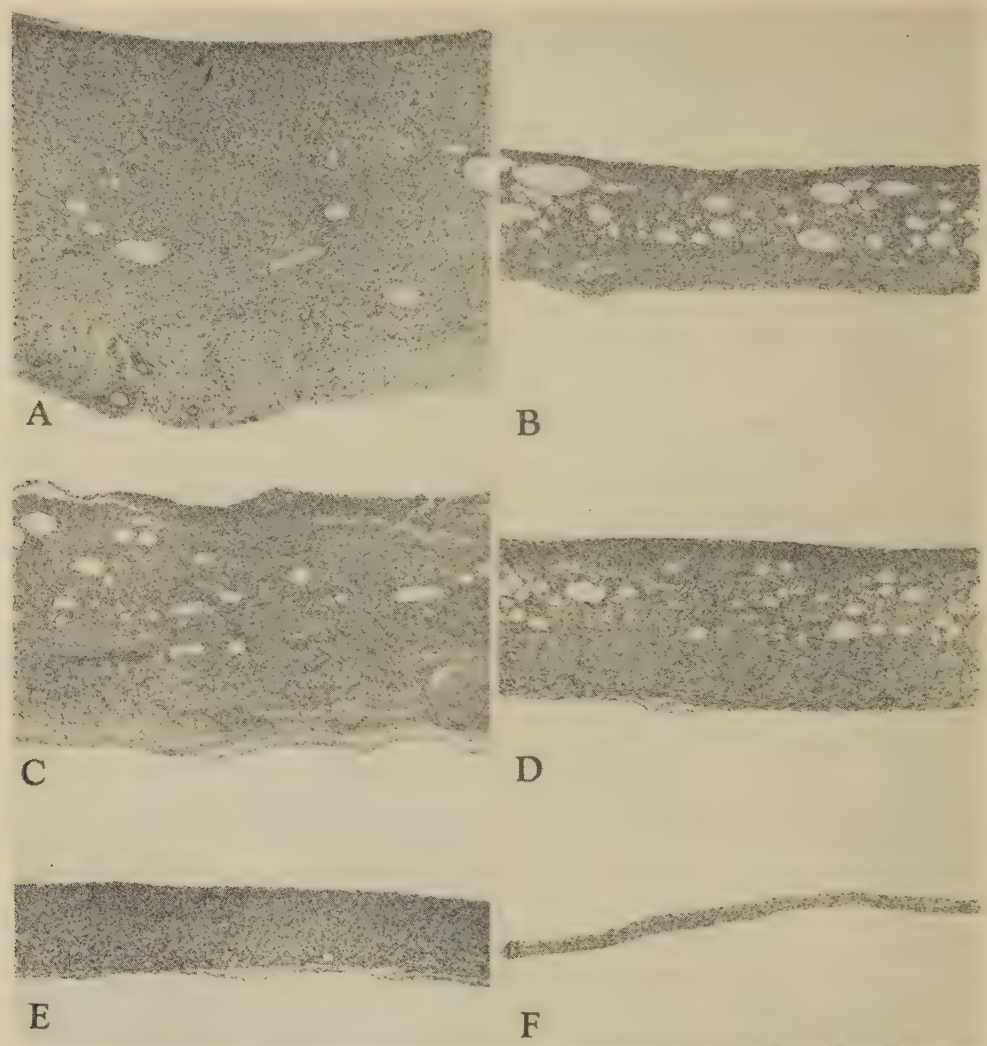

FIG. 4. Histologic appearance of "granuloma pouch" wall as influenced by various steroids. A and B: Rat No. 1 of $\triangle^5$–pregnenolone series. C and B: Rat No. 1 of DCA series. E and F: Rat No. 1 in hydrocortisone treated group of first experiment. In all instances proximal wall (left) of pouches, that which rests on spinal column, is thicker than the peripheral portion (right), situated under the skin. Everywhere the walls consist of a rather fibrous granuloma tissue. In rats treated with DCA or pregnenolone (as in untreated controls, not shown here), fibroblasts and fibers of connective tissue are well developed, the capillaries are dilated and the granulomatous tissue contains numerous sudanophilic, presumably oil-containing, vacuoles, which appear here as empty holes. On the other hand, the hydrocortisone treated animal's pouch is thin; it consists of rather atrophic connective tissue and small fibroblasts with narrow capillaries and contains no fat vacuoles.

tected by hydrocortisone against the irritating action of the croton oil solution which was in contact with them for as long as 2 weeks.

A *3rd experiment* was then conducted essentially in the same manner as the first and second, except that here 1 mg of desoxycorticosterone acetate (DCA) micro-crystals and 1 mg of $\triangle^5$—pregnenolone, respectively, were injected into the pouch wall in the experimental animals of Groups II and III, respectively. Here again Group I acted as untreated controls. Unlike in the first 2 experiments, all the animals of this third series were killed on the 9th day, by which time it had

become obvious, from mere macroscopic inspection, that these steroids fail to inhibit the inflammatory reaction when administered at the same dose level as the hydrocortisone acetate (Fig. 4 and Table III).

Table III indicates that, at this dose level, neither the formation of exudate, nor the development of the granuloma-pouch wall were significantly altered by these steroids. In this 9-day experiment, irrespective of treatment, the volume of the residual air is higher in all groups than in the first two 14-day experiments.

These findings, concerning the predominantly exudative, hemorrhagic response to croton oil, essentially confirm earlier animal experiments on the effect of gluco-corticoids upon inflammatory reactions in general(3-6). However, the use of a new technic permitted the experimental production of an inflammatory focus whose initial size and shape are always identical. It also made possible the objective, quantitative, separate determination of exudate and granuloma tissue formation, the recovery of the eliciting irritant and the accurate introduction of hormone preparations into the granuloma wall proper.

Summary. 1. Using the "granuloma pouch" technic, it was possible to produce very large amounts of inflammatory tissue and fluid (corresponding, in some cases, to as much as 25% of total body weight) in the rat, without interfering with the general well-being of the animal. 2. The introduction of small amounts of hydrocortisone, directly into the wall of the pouch, completely prevents the development of these inflammatory structures. 3. Introduction of the same amount of hydrocortisone into the lumen of the pouch, or into connective tissue at a distance of only about one mm from the pouch, is virtually ineffective. 4. The croton oil used to produce these granuloma pouches retained part of its irritating effects, even after a sojourn of 14 days, in hydrocortisone protected connective tissue. Although here it failed to cause inflammation, it still produced considerable damage, with hemorrhagic necrosis, when it was introduced into the unprotected connective tissue of other recipient animals.

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Effect of X-Irradiation on Weight and Contents of Rat Stomach, Small and Large Intestine.* (20109)

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From Naval Medical Research Institute, Bethesda, Md.

The vulnerability of the gastrointestinal tract to ionizing radiation has long been recognized. Quastler *et al.*(1), Cronkite(2), Bond (3), and others, have demonstrated the importance of the relationship of gastrointestinal damage to early deaths in animals seen after large doses of total body irradiation. Even with relatively low doses, the extreme sensitivity of the system to radiation has been demonstrated by certain functional changes, such as increased tone and amplitude of contractions of the rat intestine which occurred during irradiation or immediately thereafter, followed by a period of atonicity and lack of motility lasting for several days(4). Pharma-

* The opinions or assertions contained herein are the private ones of the author and are not necessarily to be construed as official or reflecting the views of the Navy Department or the naval service at large.

TABLE I. Effect of 500 r X-irradiation on (a) Weights of Stomach, Small and Large Intestine of the Rat, and (b) Contents of These Components.

Days after irradiation	No. of rats	Stomach: Mean wt, g	Stomach: % diff. from controls	Stomach: P	Small intestine: Mean wt, g	Small intestine: % diff. from controls	Small intestine: P	Large intestine: Mean wt, g	Large intestine: % diff. from controls	Large intestine: P
					(a) Wt of organs					
Controls	13	1.034			4.804			2.049		
1 – 2	10	.888	—14.1	<.01	3.458	—28.0	<.01	1.586	—22.6	<.01
2 – 3	"	.872	—15.7	<.01	3.150	—34.4	<.01	1.565	—23.6	<.01
3 – 4	"	.890	—13.9	<.02	3.856	—19.7	<.01	1.832	—10.6	<.2
4 – 5	"	.799	—22.7	<.01	4.590	— 4.4	<.5	1.894	— 8.2	<.01
5 – 6	"	.901	—12.9	<.02	4.765	— 0.8	>.9	1.989	— 2.9	<.7
7 – 8	"	.987	—12.2	<.6	4.582	— 4.6	<.5	1.991	— 2.8	<.7
					(b) Wt of contents					
Controls	12	1.71			4.05			4.89		
1 – 2	10	4.65	+171.3	<.01	2.94	—27.5	<.4	3.65	—25.5	<.1
2 – 3	"	3.57	+108.3	<.05	3.06	—24.5	<.4	4.24	—13.4	<.4
3 – 4	"	2.47	+ 44.1	<.4	3.66	— 9.6	<.8	4.05	—17.3	<.5
4 – 5	"	.76	— 55.6	<.2	2.96	—26.9	<.4	4.55	— 7.0	<.7
5 – 6	"	3.91	+128.1	<.01	6.22	+17.5	<.3	6.75	+37.9	<.02
7 – 8	"	3.91	+128.0	<.02	5.56	+13.0	<.4	5.35	+ 9.2	<.6

cological studies of these changes indicated that an autonomic imbalance of the enteric nerve elements of the gastrointestinal tract was induced by radiation. Such functional changes are no doubt related to the early post-irradiation gastrointestinal symptomatology such as anorexia, weight loss, diarrhea, etc.

On the basis of histo-pathological observations it is generally conceded that the small intestine is the most sensitive part of the gastrointestinal tract to ionizing radiation(5, 6). In recent experiments(7) the author has noted a marked weight loss of the small intestine of the rat following low lethal doses of x-radiation. In the present study, the relative sensitivities to radiation of the stomach, small intestine and large intestine of the rat are expressed quantitatively in terms of relative weight changes produced in these components by single acute doses of x-irradiation. In order to evaluate the role of edema or dehydration, water content of the tissues was also studied. In addition, changes in weight and character of the contents of the various components of the gastrointestinal tract were noted for correlative purposes.

Materials and methods. Relative changes induced by 500 r whole body x-irradiation in weight, amount of tissue water, and weight of the contents of the stomach, small intestine and large intestine, were studied in male Sherman strain rats, weighing between 150 to 200 g. Ten animals were sacrificed daily on the 1st, 2nd, 3rd, 4th, 5th and 7th post-irradiation days. Thirteen comparable, unirradiated animals served as controls. Animals were irradiated in aluminum cages, 3 to a cage, and placed above a standard 50 cm therapy cone, such that the beam passed anterior-posteriorly. Radiation factors were: 200 kv.p; 25 ma; cone size, 20 x 20 cm; H. V. L., 0.82 mm cu; filters, .25 mm cu 1 mm al; backscatter material, .75 cm masonite; target to skin distance, 50 cm; dose rate, 44.0 r per minute (500 r was approximately an LD_{30}—30 days). Body weights were recorded before and at intervals after irradiation. Data on body weights were combined with data from a previous experiment(7) using similar animals under identical radiation conditions (total of 82 animals). Determinations of weights of organs, of gastrointestinal contents, and water content of tissues, were made in the following manner: The animals were killed by a blow on the head at approximately the same time each day. (Since the rat is a nocturnal feeder they were killed in the morning in order that the gastrointestinal contents would be more comparable.) The gastrointestinal tract was stripped free of mesentery and removed at the esophageal-cardiac and ano-rectal junctions. The gastrointestinal tract was then

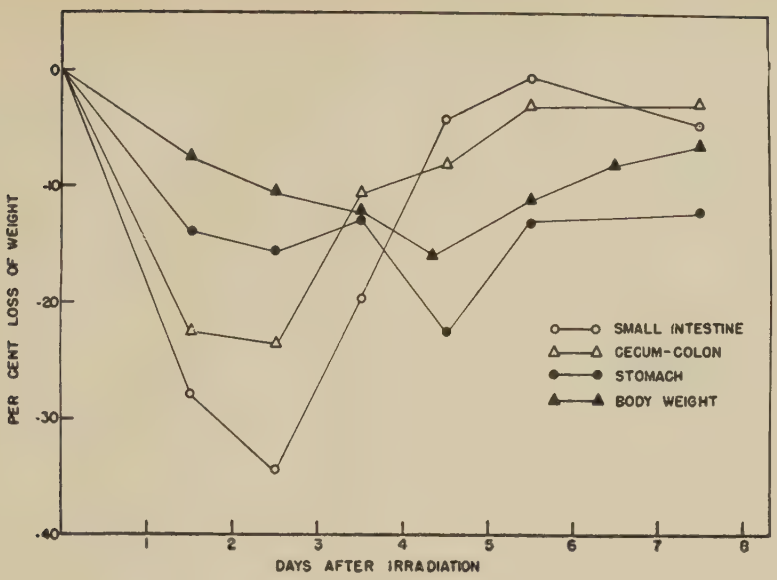

FIG. 1. Effect of 500 r X-irradiation on weights of components of gastrointestinal tract of the rat.

separated into 3 components, the stomach, the small intestine, and large intestine (cecum-colon), by severing at the pyloric sphincter and ileocecal junctions, respectively. Each component with contents was then weighed. The parts were cleaned thoroughly with water, blotted free of excess moisture and wet weights were obtained. The parts were then dried in a 100°C oven for 18 hours. (This proved to be adequate to achieve constant weight.) Water content was expressed as per cent wet weight. The mean of these values for each category was compared to the unirradiated control mean values.

Results and discussion. The changes in weight of the 3 parts of the gastrointestinal tract of the rat following 500 r x-irradiation are shown in Table I and represented graphically in Fig. 1. Although all 3 components showed depressed weight during the first 4 days after irradiation, the small intestine showed considerably greater weight loss than did the stomach or large intestine.† It is believed this greater weight loss of the small intestine reflects its greater sensitivity to radiation. Though the small and large intestines returned to approximately normal weight by the 4th and 5th days, it is interesting that the weight of the stomach did not return to normal during the course of the experiment.

Body weight loss was not nearly so extreme as gastrointestinal weight loss (Fig. 1). Following irradiation, the animals showed a steady loss of weight, reaching 16% below pre-irradiation weight on the 4th day. This was followed by a slow gain in weight, pre-irradiation weight being attained by the 11th day. Body weight changes during the early post-irradiation period might have been largely a reflection of the state of the gastrointestinal tract. Smith(8) and Nims *et al.*(9) showed that irradiated starved rats lost the same body weight as starved rats without irradiation. In irradiated rats that had access to food, however, changes in weight occurred which were dose dependent (up to 600 r)(9). The weight changes were probably related in part to the degree of anorexia with resulting lowered food intake. The anorexia was probably largely a reflection of the degree of damage of the gastrointestinal tract.

The etiology of the acute changes in weight of the components of the gastrointestinal tract are no doubt quite complicated. The variations in weights were not due to changes in water content (edema or dehydration) of these tissues since the water content remained

† $P < 0.01$ by t-test on 2nd day.

fairly constant throughout the period of observation, with no marked variation from the controls. The variation in mean per cent water of the tissues was as follows: stomach, controls, 77.7; irradiated, 77.0-78.1 (mean 77.5); small intestine, controls, 79.7; irradiated, 79.2-80.6 (mean 79.4); large intestine, controls, 79.9; irradiated, 79.2-81.0 (mean 80.1).

Reduced food intake occurring during the first few days would not likely account for all of the weight loss. In a previous experiment (7) it was shown that in rats starved to lose the same body weight as irradiated rats, the small intestine did not show as great a weight loss as did the small intestines of the irradiated animals. Therefore, aside from nutritional effects, some of the weight loss must be accounted for on the basis of specific changes induced by irradiation. No doubt much of the weight loss may be accounted for on the basis of destruction and failure of normal replacement of the sensitive epithelial and lymphoid cells. Epithelial cell destruction occurred primarily during the first 24 hours with this dose of radiation(7). Leblond and Stevens(10) have shown that the intestinal epithelium normally replaces itself about every 1½ days. Since radiation produces mitotic inhibition of these epithelial cells(11-13), failure of normal replacement of the epithelium becomes an important factor in weight loss.

The contents of the gastrointestinal tract showed considerable variation during the 7-day post-irradiation period (Table I). The marked increase in contents of the stomach during the first 3-4 days demonstrated clearly the radiation-induced emptying defect of the stomach, which has previously been reported (14-16). Increased tone of the pyloric sphincter together with the atony of the stomach might well have accounted for this condition. During the 3-4 day period the contents of the small and large intestines became decreased slightly and liquid and foul-smelling in nature. These changes coincided with the time when the gross appearance of the intestine was worst, *e.g.* atony, dilatation and lack of motility.

By the 4th-5th days, the contents of the entire gastrointestinal tract assumed a normal, semisolid state. The increased contents noted during this time probably represented increased food intake to compensate for the earlier period of lowered food intake. At this time, also, the weight of the gastrointestinal tract and the gross appearance returned toward normal.

The acute fluctuations of weight and contents of the gastrointestinal tract were part of an over all complicated reaction of that system to radiation that occurred during the early post-irradiation period. Thus, after 500 r in the rat the first 3-4 days were characterized by morphological and functional changes associated with the gastrointestinal tract, among which were: damage to the epithelial and lymphoid elements; loss of weight of the gastrointestinal tract, particularly of the small intestine; a short-lived increased intestinal motility followed by depressed motility; reduction in activity of the enzyme cholinesterase(7); anorexia with lowered food intake and loss of body weight; and the development of abnormal liquid contents of the intestine with diarrhea (in some animals). The period from the 3rd and 4th day to the 7th day was marked by recovery which began rather abruptly about the 3rd or 4th post-irradiation day. There was no further evidence of epithelial damage, gastrointestinal weights returned nearly to normal, motility appeared normal, the enzyme cholinesterase was slowly increasing, the animals began to eat normally and to gain weight, diarrhea disappeared and the contents became normal in appearance.

The gastrointestinal changes reported in this paper apply to the 500 r x-ray dosage used under the conditions of this experiment. Many of the phenomena are dose dependent, *e.g.*, intestinal weight loss(17). With larger doses many of the symptoms and signs may be aggravated and with smaller doses they may be less prominent.

Summary. 1. The effect of 500 r whole body irradiation on organ weights and weights of the contents of the stomach, small intestine and large intestine of the rat was studied. All three components showed loss of weight during the first 3- to 4-day post-irradiation period which was greater than body weight loss. The stomach, small intestine and large intestine

showed percentage weight losses of 15.7, 34.4 and 23.6, respectively. Organ weights, except for the stomach, returned nearly to normal on the 4th and 5th day. The significantly greater weight loss in the small intestine was believed to reflect greater sensitivity of this part to ionizing radiation. It was pointed out that these weight changes were not due to dehydration or edema of the tissues or to the lowered food intake alone, but were in a large part due to specific effects of radiation. 2. The stomach showed the greatest variation in contents after irradiation, with a marked increase occurring during the first 72 hours which was believed to be associated with delayed emptying of the stomach. During this same period the small intestine and large intestine showed slightly reduced contents which became liquid and foul-smelling in character. From the 3rd to 4th days through the 7th day there was a marked increase in contents in all parts above the control range with a change to the normal semi-solid state. It was believed that this change was the reflection of compensatory increased food consumption associated with improved gastrointestinal function.

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Certain Nucleotide Relationships in Normal and in Tumor-Bearing Mice.* (20110)

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We have reported that male mice bearing implanted sarcoma 180 undergo a significant lowering in blood adenosine triphosphate during the interval of tumor growth(1). The first part of the present investigation was concerned with ascertaining whether similar alterations in blood nucleotide appear in female mice during the estrous period as compared with the diestrous, or during pregnancy when extensive mitotic activity occurs which may be similar to that of tumor growth. At the same time, analyses were performed on the blood of

* The work reported in this paper was supported in part by grants from the U. S. Public Health Service on recommendation of the National Advisory Cancer Council.

animals fed with thyroid or with thiouracil to determine whether the marked alterations in tissue metabolism induced by these agents affect the nucleotide pattern of the blood.

In addition, it appeared desirable to determine whether the lowered adenosine triphosphate level occurring in the blood during tumor growth is related to the nucleotide levels in the tumor tissue itself. Another point appeared to merit investigation: one of us(2) had reported that the injection of 5-adenylate into human subjects elevated the adenosine triphosphate of the blood. The question arose as to whether similar increases could be produced in mice, especially in the blood of tumor-bearing mice, as well as in the tumor tissue of such mice.

Materials and methods. White Swiss female mice of 20-22 g were used throughout, except for the experiments on pregnant mice where body weights were undetermined. The state of the estrous cycle was ascertained by the use of a standard vaginal smear method. For the metabolism experiments, mice were fed for periods of between 13 and 23 days on Rockland diet containing 2% desiccated thyroid or 0.5% thiouracil powder. In the case of the tumor experiments, mice were implanted bilaterally with sarcoma 180, using methods now standard for this procedure. The removal of blood, the preparation of filtrates, and the measurement of total purine and known nucleotides was carried out as before(1). For tumor removal, the animal was killed by cervical fracture; then as quickly as possible the tumor tissue was excised, the central necrotic core of each tumor mass removed and discarded, and the remaining viable tissue dropped into liquid nitrogen. Enough tumor tissue was taken, often from multiple donors of the same experimental class, to yield an aliquot of about 1 g of tissue. The frozen fragments were pulverized and extracted once with a 5 ml aliquot of cold 10% trichloracetic acid and twice with 2 ml aliquots of 5% trichloracetic acid. The extracts, following centrifugation, were combined, neutralized, made to a 10 ml volume, treated with 0.5 ml 25% barium acetate and 4 volumes of ethyl alcohol, and placed in the cold room overnight. The barium precipitates were collected by centrifugation, dissolved in dilute HCl, freed of barium with Na_2SO_4, neutralized, made to volume and assayed enzymatically for adenine due to adenylic acid (AA), adenosine diphosphate (ADP), and adenosine triphosphate (ATP) in a fashion similar to that used for blood.

TABLE I. Known Blood Nucleotide (Combined AA, ADP, and ATP) and Total Blood Purine for Normal Male Mice, Female Mice in Estrus, Diestrus, Pregnancy, and after Feeding on Diet Containing Thyroid and Thiouracil. The values are means and their stand. errors, expressed as mg % adenine. Also shown are number of determinations (n) used in calculating the means and stand. errors.

Condition	n	Known nucleotide	Total purine
Males, normal	39	7.0 ± .20	13.3 ± .27
Females, estrus	21	6.8 ± .17	13.4 ± .28
" diestrus	24	7.0 ± .13	13.5 ± .28
" pregnant	27	5.8 ± .22	12.5 ± .27
Thyroid fed	13	7.2 ± .27	14.3 ± .32
Thiouracil fed	13	7.5 ± .20	14.1 ± .29

TABLE II. AA, ADP, and ATP, and Total Purine Content of Normal Blood and Blood from Tumor-Bearing Mice, Sacrificed at Various Intervals after Intravenous Injection of AA as the Sodium Salt. Shown in the last column is the change in proportion of known nucleotide as a function of the total purine. Values expressed as mg % adenine.

Condition, mg AA	hr	ATP	ADP	AA	Sum (AA, ADP, ATP)	Total purine	% known nucleotide of total purine
Normal (control)		6.8	.4	.0	7.2	13.2	54.6
4	½	10.0	.2	.5	10.7	15.1	70.8
4	1	8.5	.7	.5	9.7	14.0	69.2
4	3	6.8	.0	.0	6.8	12.4	53.2
4	4	7.1	.3	.1	7.5	13.9	53.9
8	½	12.5	.6	.3	13.4	19.2	69.7
7-day tumor		5.0	.3	.3	5.6	11.5	48.7
4	1	7.9	.5	.4	8.8	13.4	65.7
4	4	4.4	.0	.4	4.8	10.6	45.3

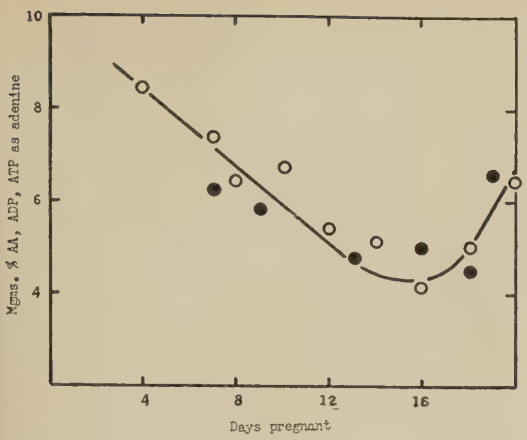

FIG. 1. Known nucleotide (AA, ADP, and ATP) in blood of mice at various stages of pregnancy. Two sets of assays indicated.

Results and discussion. In Table I are presented the mean values along with their standard errors of combined known blood nucleotides (AA, ADP, ATP) and the total purine for female mice during estrus, diestrus, pregnancy, and for animals fed on a diet containing thyroid or thiouracil. Also shown as the first entry in Table I are data taken from our earlier publication(1) on the blood of normal males. As we had found earlier, the major part of the adenine in the known nucleotide fraction is present as ATP (see control line in Table II). Statistical analysis reveals that the blood values shown in Table I do not (except for those of pregnant animals) differ significantly from the values obtained earlier for normal males. The values for pregnant mice compared with females in either estrus or diestrus show a significant reduction in known nucleotide (the difference between the means exceeds their standard errors by more than 4 times), as well as in total purine ($d/S.E._m$—2.6). The animals of the pregnancy group ranged from 4 to 19 days of term. Examination of the individual data shows that the greatest reduction in known blood nucleotide occurs at about the 16th day of pregnancy. The pertinent data are plotted in Fig. 1, representing 2 separate runs made several months apart. It is clear from these results that depression in blood nucleotide is not specific to the presence of sarcoma 180 in the animal. The presence of a growing fetus brings about a similar alteration, although not as marked a one. In the pregnant animal, the level begins to return to normal after about the 16th day (5 days before parturition), whereas in the tumor-bearing animal, the level does not begin to rise until the time when involution and resorption of the tumor often begin (about the 12th day after implantation).

In Fig. 2 are shown data on the known nucleotide content (AA, ADP, ATP) of tumor tissue expressed as mg of adenine/100 g of tissue. The figures alongside each point represent the number of separate analyses performed. Also plotted are the data from our earlier publication(1) on the blood level of these nucleotides in mice in which tumors are growing. It is clear that the decrease in blood nucleotide is related to the increase in concen-

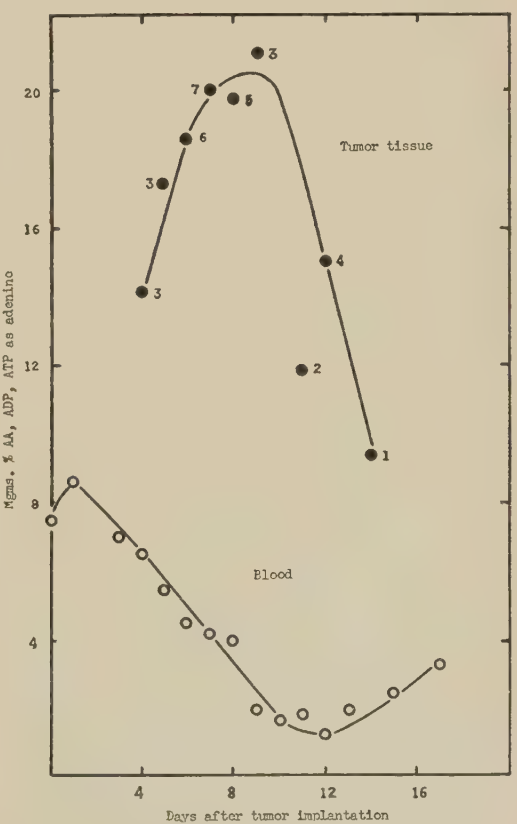

FIG. 2. Known nucleotide (AA, ADP, and ATP) in blood and in tumor tissue of mice at various stages following tumor implantation. Figures in upper curve represent No. of separate analyses performed.

TABLE III. AA, ADP, and ATP, and Total Purine in 7-8 Day Tumors, following Intravenous Injection of 4 mg of AA. Animals sacrificed 1 hr after inj. Values expressed as mg % adenine.

Animal No.	AA	ADP	ATP	Total purine
		Controls		
E62	3.7	.0	11.6	38.6
63	4.2	.9	7.4	32.0
67	3.0	4.8	7.9	33.7
80	3.3	.5	13.0	33.3
82	3.4	3.2	8.3	32.9
85	2.3	.0	8.5	25.8
86	2.2	2.3	7.8	27.2
Mean	3.1	1.7	9.2	31.9
		AA injected		
E64	2.5	3.5	6.9	27.3
65	1.6	6.3	4.2	26.2
66	2.2	6.7	5.2	31.0
81	1.8	1.9	2.3	17.4
83	2.3	2.9	9.9	28.5
84	.9	1.2	3.3	15.0
Mean	1.9	3.8	5.3	24.2

tration of tumor nucleotide, and that the blood level does not begin to rise again until its concentration in the tumor has decreased appreciably. Tumor nucleotide reaches its highest concentration at about the 8th day after implantation. This corresponds to the period when the proportion of viable cells in the tumor is at a maximum(3).

The next part of the study was concerned with the effect of injected adenylic acid on the blood nucleotide level of normal and tumor-bearing mice. In normal humans, the injection of 20 mg of 5-adenylic acid intramuscularly produces a transient increase in the blood ATP level without any appreciable increase in the total purine(2). Intravenous injection of AA was not performed on human subjects. In the mouse experiments reported here, 4 (and in one case 8) mg doses of AA as the sodium salt in aqueous solution† were injected intravenously. Animals were sacrificed at the end of 30 minutes, one, 3, and 4 hours, and the blood assayed for AA, ADP, ATP, and total purine. The results are shown in Table II. It is clear that the injection of AA produces a marked elevation in the blood ATP of normal mice. This increase persists for about an hour and then the level returns to normal. The increase in human blood ATP after intramuscular injection of AA is not usually seen until one hour after injection. At the 4 mg dose, it is interesting to point out that for normal mice the increase in ATP is greater than that which occurs in the total purine, offering additional evidence that injected AA causes, at least in part, a shift in the purine reservoir in the direction of ATP. This is also shown by the figures in the 6th column of Table II, where the percent of known nucleotide, as a function of total purine, is shown to rise and then return to normal by the end of 3 hours. Similar results have been obtained for the blood of tumor-bearing animals. These data are shown in the lower section of Table II.

In a preliminary series of experiments on tumor-bearing animals from which blood had been taken for the analyses described above and which were injected intravenously with 4 mg of AA, low values for known nucleotides in the tumor tissue were observed. The greatest effect appeared to be on tumors which had been implanted for about 7-8 days. Tumor tissue analyses, accordingly, were carried out mainly on such animals, both with and without injected adenylic acid. The results, shown in Table III, indicate that whereas injected AA elevates the level of blood ATP, it lowers the ATP level in the tumor tissue. It appears also to lower the AA and the total purine level in the tumor, but to raise the ADP. The lowered AA and ATP and the elevated ADP levels seem to suggest an effect of the injected nucleotide on the myokinase system:

$$2\text{ADP} \underset{}{\overset{\text{myokinase}}{\rightleftharpoons}} \text{AA} + \text{ATP}$$

Summary. 1. Data are presented on the blood nucleotide level of mice in estrus, diestrus, in pregnancy, and after thyroid and thiouracil feeding. Of these conditions, only pregnancy produces a significant lowering of the nucleotide fraction containing AA, ADP, and ATP. This decrease is similar to that observed in mice bearing sarcoma 180. In pregnant mice the blood level begins to return to normal after the 16th day of pregnancy. 2. During tumor growth, as blood nucleotide

† The adenylic acid used was a gift from the Ernst Bischoff Co. It is marketed under the trade name of My-B-Den.

decreases, tumor nucleotide increases, reaching a maximum at about the 8th day after implantation, followed by a rapid fall. This fall appears to be correlated with the tendency (beginning about the 10th day of tumor growth) for the blood nucleotide level in tumor-bearing mice to return to normal. 3. The intravenous injection of adenylic acid into normal mice causes a transitory rise in blood ATP. With a dose of 4 mg, this increase is in excess of that in the total purine. A similar increase in blood ATP occurs after the injection of AA into the blood stream of tumor-bearing animals. The tumors of such injected animals show a lowered level of AA, ATP, and total purine, but an elevated level of ADP as compared with uninjected controls.

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Single Pyramidal Fiber Responses to Electrical Stimulation.* (20111)

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In an earlier study of the responses of spinal motor neurones following selective activation of pyramidal fibers, an explanation of the findings was based upon the assumption that the pyramidal fibers responded once to each electrical pulse and that there was numerical agreement between the number of pyramidal shocks and the number of impulses initiated in each of the responding fibers(1). Two deviations from the numerical equivalence were considered possible. Some pyramidal fibers may have behaved like certain fibers of peripheral sensory nerves and responded repetitively to single shocks of long duration(2,3). Other pyramidal fibers, due to their presumed longer refractory periods, may have been unable to follow the higher stimulation frequencies, with the resulting occurrence of a response deficit.

It was the purpose of this study to test this assumption by recording responses from single pyramidal fibers activated by pulses of the parameters used previously.

Methods. The experiments have been performed upon cats and macaques in surgical anesthesia as a result of the administration of diallylbarbituric acid in urethane solution. In some instances movement was prevented through the use of d-tubocurarine coupled with artificial ventilation at a level roughly equivalent to the level of ventilation established by the preparation before curarization. The dorsal surface of the medulla oblongata was exposed through an enlarged foramen magnum and the spinal cord was exposed by laminectomy at the desired level. *Stimulating pulses* were controllable in all dimensions and were triggered by the oscillograph sweep generator. The rectangular pulses were applied to the preparation through the mediation of a Grass Stimulus Isolation Unit which slowed the rise and decay times to a degree insignificant for the purposes of this study and which permitted the maintenance of a steady voltage at the stimulating electrodes throughout the duration of the pulse. The current through the stimulating electrodes was measured with the aid of a monitoring oscillograph. The stimulating pulses were applied to the preparation through two forms of stimulating electrodes. Orthodromically conducted volleys were initiated at the level of the pyramid through concentric bipolar electrodes oriented stereotactically. Antidromically conducted volleys were initiated at appropriate cord

* With the assistance of grants in aid of research from the Rockefeller Foundation and the National Institutes of Health of the U. S. Public Health Service.

levels through pairs of fine insect needles thrust into position in the lateral column. The needles were insulated except at the tips, held parallel and 1-2 mm apart by a small cardboard spacer. *Recordings* were made photographically from a cathode ray oscilloscope activated by a capacitance-coupled pre-amplifier. The recording electrodes were 12.5 micron insulated alloy wires used either singly or cemented together to form bipolar pairs(4). These could be handled with plastic-tipped forceps and used to explore for suitable positions from which to record single fiber responses. *Impulses recorded from microelectrodes* in the region of the pyramidal decussation as a result of stimulation in the spinal cord could have been the result of activation of systems other than the corticospinal system. In experiments involving antidromic activation, this possibility was controlled in the following way. Bipolar concentric recording electrodes were introduced into the pyramid. The contralateral lateral column was then explored, using the bipolar stimulating needle electrodes with the tips oriented cephalocaudally. At lower cord levels it was found that the stimulating electrodes must be placed with great precision in order to initiate a response which could be recorded from the bipolar concentric electrodes in the pyramid. Movement of the stimulating electrodes through a distance of a millimeter or less was sufficient to eliminate the pyramidal response. Using this type of control continuously as a monitor for the remainder of the experiment, it was found that the single fiber responses recorded from the microelectrode over a second channel always fell within the temporal limits of the summated pyramidal response.

Results. Numerous unsuccessful attempts were made to record responses in single pyramidal fibers after orthodromic conduction. It was not at all difficult to record summated volleys from the corticospinal tracts at cord levels after stimulation of the bulbar pyramid. The failure to record single fiber responses apparently depended upon two sources of difficulty. In the spinal cord the fibers of the lateral corticospinal tracts form a fairly compact bundle. That this is true, even at lumbar levels where the number of fibers is relatively small, is attested to by the fact that the recording electrode may enter and leave the regions giving rise to summated volleys with movements of less than a millimeter. When such a compact group of fibers is activated synchronously, the 12.5 μ electrode does not have sufficient resolving power to isolate responses from single members of the population. In addition, confusion may arise from the fact that the lateral corticospinal tract lies in close approximation to cells of the spinal gray matter which respond to corticospinal impulses(5). In those instances in which single unit records were derived from spinal cord placements, later histological examination of electrode positions confirmed the suspicion that such records were probably derived from cells of the spinal gray rather than from fibers.

An attempt was then made to circumvent these two difficulties by recording from pyramidal fibers after antidromic conduction from spinal cord levels to the level of the pyramidal decussation. With this form of experiment the possibility of recording from synaptically activated cells would be greatly reduced. The intermingling of fibers from the two sides coupled with the longitudinal dispersion of the decussating fibers would reduce the concentration of the active population at any one locus and afford a better opportunity for selecting a single responding fiber.

Responses in single pyramidal fibers have been recorded after antidromic conduction in seven cats and one macaque. The conduction velocities of all 14 of the fibers studied have fallen in the range from 35 to 75 meters per second. Although it is impossible to estimate the diameters of these fibers from their conduction velocities with any degree of accuracy (6), it is probable that they fall into the group of medium-to-large pyramidal fibers and very doubtful that any of the numerous small pyramidal fibers are represented in this group.

Illustrations of responses typical of such fibers are presented in Fig. 1. At this single placement in the pyramidal decussation, activity was recorded from two different fibers. The indicated conduction velocity for the larger, faster response is just short of 55 m.p.s., while that for the smaller, slower response is 38 m.p.s. With a current intensity 5 times

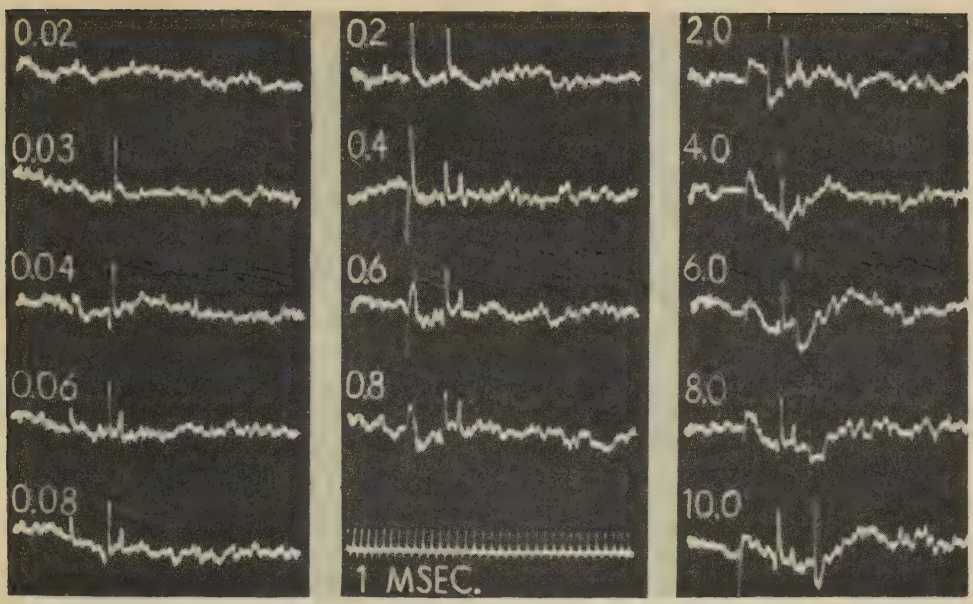

FIG. 1. Single pyramidal fiber responses in the cat recorded from the pyramidal decussation following stimulation of lateral corticospinal fibers at the level of L6. The current intensity was 5 times the rheobase for the faster of the two fibers responding. The duration of the stimulating pulse is indicated for each trace. The faster of the two fibers responded first at a duration of 0.03 msec.; the slower responded first at a duration of 0.06 msec. The "off" shock artefact first becomes clearly evident at 0.2 msec. and is seen at successively later positions in the following traces. At 4.0 msec. it almost overlies the response of the faster fiber and at 6.0 interfered with the recording from the slower of the two fibers.

greater than rheobasic strength, the faster of these two fibers responded first to a pulse of 0.03 msec. duration. As the duration of the stimulating pulse was increased, no indication of repetitive firing on the part of this fiber is evident from the record. The response of the second, slower fiber first became evident at a pulse duration of 0.06 msec. That this is not a second response from the same fast fiber is an opinion based on the lower amplitude of the response, on the uniformity of its latent period throughout the series, on the all-or-none nature of the responses and on the fact that the configuration did not repeat even though the duration of the pulse was increased beyond the illustrated 10 msec.

Four of the single fibers from which records were made showed responses initiated by the "off" transient of the stimulating pulse. This form of repetitive activity was seen only when the pulse duration was in excess of 4.0 milliseconds. No off-responses were recorded at shorter pulse durations.

When tested with pulse durations of 0.5 msec. all of the fibers studied responded faithfully and without response deficits to frequencies of stimulation up to 500 per second.

Discussion. It might be considered dangerous to base conclusions on results involving antidromically conducted impulses since this is an abnormal direction of conduction. However, there is no evidence that excitation and conduction characteristics undergo any alteration along the length of a nerve fiber as long as its structural characteristics remain uniform. It has been found that the conduction velocity of this system of fibers is uniform over its extent in the white matter of the spinal cord(6). On the other hand, the formation of collaterals constitutes the most probable explanation for observed changes in conduction velocity in the dorsal column system of fibers(7). It is therefore considered reasonable to assume that characteristics defined on the basis of antidromic conduction bear a close resemblance to the normal.

The possibility of repetitive responses on the parts of nerve fibers activated by long

duration pulses must be taken seriously in the light of the findings of Skoglund and others (2,3) that peripheral afferent fibers show repetitive responses to rectangular and to linearly increasing currents at intensity levels which are not far in excess of the rheobase. It is not clear whether the likelihood of repetitive firing is equally great for both pulse forms. Neither is it clear whether the probability of repetitive firing is different for fibers of different structural characteristics.

The results of the present study justify the assumption of a one-to-one relationship between stimulus to and response of single pyramidal fibers within the limits of the stimulus parameters used in the previous investigation. Unfortunately, the evidence presented can be applied only to fibers conducting at velocities in excess of 35 meters per second. These are in all likelihood those fibers which have diameters above the median value. Thus we must remain in ignorance about the behavior of the great number of smaller fibers which are found in the medullary pyramid.

Summary. 1. Responses of single pyramidal fibers to stimulation with rectangular electrical pulses of varying parameters have been studied in the cat and the macaque. 2. Those fibers conducting at velocities in excess of 35 m.p.s. do not respond repetitively to pulses of less than 4.0 msec. in duration. Some fibers have shown off-responses to pulses in excess of this duration. 3. All fibers studied have followed repetitive stimulation up to frequencies of 500 per second without response deficit.

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Electron Micrographs of Motor End-Plates.*† (20112)

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The purpose of this paper is to report some preliminary observations on the motor end-plates as seen in electron micrographs. The present concept concerning the nerve muscle relationship at this point is that there is no direct connection between them. Impulses are thought to be transmitted from nerve to muscle at this site by a humoral substance, inorganic ionic changes, or by the action of end-plate potentials(1). However, the possibility of some more or less direct morphological relationship existing between nerve and muscle at this juncture, such as the "periterminal net" of Boeke(2), while discredited, has never been disproved(3).

Material and methods. The material for this study consists of the motor end-plates of the intercostal muscles of the rat. The gold chloride method used for the demonstration of the end-plates was that described by Warren(4). Further treatment of the tissue for electron microscope study consisted of bleaching the gold impregnated pieces of muscle and associated end-plates in a solution of 0.5% potassium cyanide in 50% ethanol. This process was watched under the dissecting microscope so that it could be stopped at the

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FIG. 1, 2, 3, 4, and 5. Whole mounts of motor end-plates showing the variation in size and distribution of the nerve endings within the sole-plate sarcoplasm. Small granules may be seen (Fig. 1-2) in the sole-plate sarcoplasm between the branching nerve endings.

FIG. 6. Electron micrograph of section through motor end-plate. An apparently close relationship of the Z membranes of the muscle and the nerve endings is seen at A and B. The dark irregular mass at C is thought to be an artifact (excessive gold deposit). Cross sections of neurofibrils appear in the upper cross section of nerve ending.

FIG. 7. This section shows a layer of sarcolemma between the end-plate and muscle fiber. Here some of the Z membranes may be seen to penetrate the overlying sarcolemma layer and extend well within the sole-plate sarcoplasm (D and below). The object at E is unidentified. It may represent the space between two adjacent myofibrils.

proper stage of differentiation by placing the pieces in 70% ethanol. Further preparation of the tissue was made according to the method of Beams, *et al.*(5). The scale on the electron micrographs equals 1 μ.

Results. Fig. 1, 2, 3, 4, and 5 are whole mounts of motor end-plates that were included to show the type of preparation that was used for electron microscope study. Detailed studies on this type of preparation have been made by many and will not be further discussed here(3).

At the bottom right of Fig. 7 the sarcolemma seems to split, sending a relatively thin layer over the surface and enclosing the end-plate; the inner layer continues between the end organ and the contractile portion of the muscle. The gold impregnated cross sections

of the nerve endings are seen within the fibrous appearing sole-plate sarcoplasm. Because of the density of the impregnated nerve endings, nothing can be observed of their detailed structure. The interesting part of this figure is that the Z membranes of the muscle seem to penetrate the overlying layer of sarcolemma and to end in the sole-plate sarcoplasm. At D this condition can best be seen; but it is also evident in the Z membranes extending below the one at D. The longitudinally oriented line at E is unidentified unless it represents the space between two adjacent myofibrils.

An even more striking illustration of the penetration of the Z membranes into the sole-plate sarcoplasm is shown in Fig. 6, A and B. Although the gold impregnated Z membranes are somewhat distorted, they nevertheless can be clearly followed into the sole-plate, where they are observed to come in close association, if not in direct contact with, the nerve endings. This condition is the same as that in Fig. 7 except that here the Z membranes stand out more sharply because of being more deeply impregnated with gold. Much the same sort of general structure of the end-plate exists in Fig. 6 as in Fig. 7, except that the sarcolemma is not so evident. The body at C, Fig. 6 is thought to be an artifact due to excessive gold deposit.

In the upper cross section of the nerve ending in Fig. 6 can be seen dark irregularly arranged granular-like structures that may be only gold deposits; however, the smaller, more numerous, and less dense elements present are probably neurofibrils. The structure of the peripheral elements of the nerve endings is masked by the gold.

Discussion. Histological illustrations of the motor end-plate show it to be in direct contact with the contractile portion of the muscle(2). However, this does not always seem to be the case since sometimes a layer of sarcolemma may be seen to lie between them. The work herein reported seems to demonstrate in the motor end-plate a closer association of the Z membranes of the muscle fiber with the nerve endings than is generally thought. The only previously described type of morphological structure within the end-plate with which this condition can be compared is the "periterminal net" of Boeke(2). However, we do not think a direct morphological connection exists between the Z membranes of the muscle and the nerve endings, but rather that the relationship here between the two is comparable to that which exists at a synapse. It is conceivable that nerve impulse transmission would be facilitated more under the morphological conditions herein described for motor end-plate, whether it be by neuro-humoral substance, or by end-plate potentials, than it would be if the impulse had to pass, however the method, for relatively long distances through the sole-plate sarcoplasm to reach the underlying muscle.

Summary. The Z membranes of the underlying muscle fiber appear to penetrate the end-plate and extend for some distance into the sole-plate sarcoplasm. Here they were observed to be in close association, if not in direct contact with, the nerve elements. Such an arrangement represents a closer association of the nerve and contractile muscle elements at the motor end-plate than is generally thought to be present.

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Whole Blood Clotting, Clot Retraction and Prothrombin Utilization in Burros Following Total Body Gamma Radiation.* (20113)

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The total-body gamma radiation syndrome as seen in the burro is similar to that reviewed by Cronkite and Brecher(1) for common laboratory animals and man. The hemorrhagic phase consisted of purpura, petechial hemorrhage and pancytopenia of blood and bone marrow elements(2).

In a group of burros subjected to lethal amounts of gamma radiation a particular study was made of the clotting mechanisms, especially whole blood clotting, clot retraction and prothrombin utilization. The results of that investigation are herewith reported.

Materials and methods. Nine healthy female burros, 3 to 4 years old, were exposed to 950 r of gamma radiation, which was about 90% lethal within 30 days for burros ($LD_{90/30}$). The exposure device has been described by Wilding *et al.*(3) and the dosimetry calculated in a manner described by Rust *et al.*(4). Pre-irradiation values established each animal as its own control. Methods were controlled by concurrent tests on non-irradiated animals. Blood was collected by jugular venipuncture directly from 15 ga. needles into 4 calibrated 13 × 100 mm pyrex tubes. Exactly 4.5 ml of blood was placed in each tube, one of which contained 0.5 ml of M/10 sodium oxalate. Platelet counts were made by the direct method described by Wintrobe(5). The plasma was separated immediately from the preoxalated tube (Tube A) by centrifugation at 2000-2500 R.P.M. for 5 minutes. The 3 remaining tubes were placed in a constant temperature, 37°C, water bath. The whole blood clotting time was determined by visual examination, timing from the filling of the first tube, which took 5-10 seconds. Clot retraction was observed after one hour and was classified as "good", as seen in the normal burro blood, when the clot was surrounded by serum and had retracted from the top of the serum or the bottom of the tube; "fair", when the clot was surrounded by serum but not retracted from the top or bottom; "slight", when a small amount of serum was visible but the clot was not surrounded, or "none", when no serum was observed. Thirty minutes after venipuncture (Tube I) and 60 minutes after clotting (Tube II), 0.5 ml of M/10 sodium oxalate was added, mixed with a glass rod, and the serum-plasma removed by centrifugation as in Tube A. Immediately following separation of cells one-stage and 2-stage prothrombin determinations were performed on all samples of plasma or serum. The modified one-stage prothrombin test(6) was the mixing of 0.1 ml of test plasma (serum), with 0.1 ml of normal fresh burro plasma treated with $BaSO_4$(7) and then adding 0.4 ml of Simplastin (Chilcott Labs.). The 2-stage prothrombin procedure described by Ware and Seegers(8) was used with the following modifications. $BaCO_3$ adsorbed burro serum was used instead of bovine serum as a source of AcGlobulin. Fibrinogen was prepared by adsorbing normal oxalated burro plasma with $BaSO_4$, precipitating with one fourth saturated $(NH_4)_2SO_4$ and dialysing against saline containing 1:200 of 1.2 M sodium citrate. Defibrination was accomplished by the addition of 25 units/ml of Upjohn's thrombin. Difco 2-stage prothrombin reagent was used as the activating mixture. A comparison of prothrombin concentration in preoxalated freshly drawn blood (Tube A) with residual prothrombin in Tube I and Tube II gave a measure of prothrombin consumption, a method suggested by Buckwalter *et al.*(9) and Langdell *et al.*(10). Recalcified plasma clotting times were determined by the method described by Ferguson *et al.*(11). Assays were done in a room held at a tempera-

* The advice of Dr. J. H. Ferguson and technical assistance of Fannie H. Cross are gratefully acknowledged.

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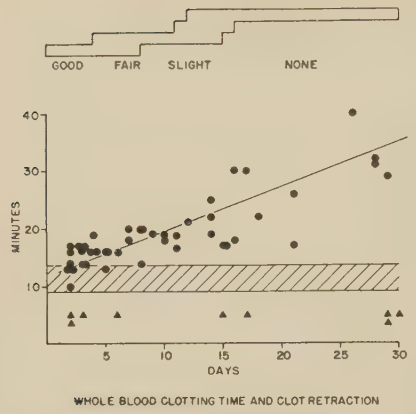

FIG. 1. Distribution of deaths represented by triangles; range of whole blood clotting time (9-13.5 min.) of pre-irradiated burros represented by shaded area; whole blood clotting times on various days after irradiation represented by dots; observations of clot retraction of blood after irradiation represented in blocked area above graph with descriptive subscripts.

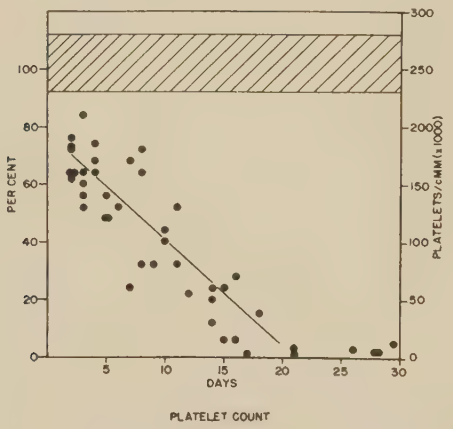

FIG. 2. Range of pre-irradiation platelet counts for burros (230000-280000/mm³) represented by shaded area. Post irradiation values on specific days of experiment represented by dots.

ture of 28°C. The straight lines on the diagrams of results were plotted by the method of least squares from all experimental data represented by dots on the diagrams and represent a trend after the initial change was noted.

Results. The *syndrome* and deaths were typical of radiation sickness in the burro (Fig. 1). The burros dying on 2nd and 3rd days had encephalitis-like symptoms. Lameness and necrotic ulcerations of mucous membranes occurred later in survivors and after 2 weeks a clinical hemorrhagic syndrome was apparent. The 3 burros living 29 to 30 days post irradiation bled through the apparently intact skin over large areas of their bodies. No gross pathological evidence of radiation damage was noted in animals dying before the 6th day. The other animals had petechiae and ecchymosis of muscles, mesentery, peritoneum and submucosa of bladder and vulva. Pulmonary hemorrhages were also observed. The lymph nodes and perinodular tissues were soft, dark and edematous without obvious hemorrhage.

There was a significant increase in whole blood *clotting times* after the 3rd post irradiation day (Fig. 1).

There was a change in the *clot retraction* rate 4 days after exposure to irradiation (Fig. 1). Gradations in quality of retraction were clearly observed.

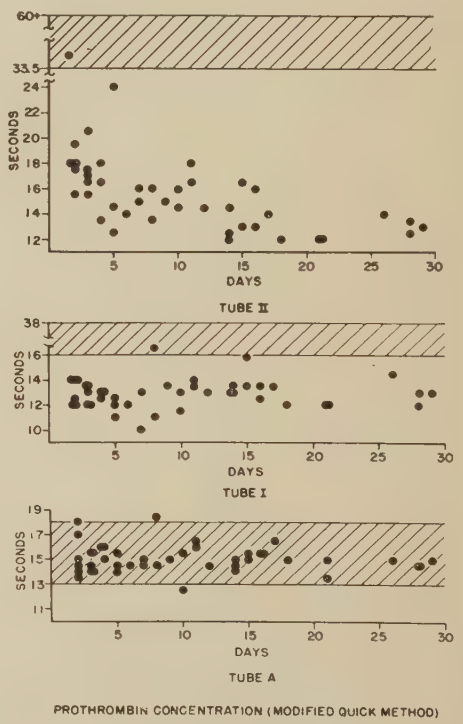

FIG. 3. Prothrombin clotting times (p.c.t.) determined by modified one-stage (Quick) method. Shaded areas represent pre-irradiation range, dots represent individual values on various days after irradiation. Normal pre-irradiation p.c.t. were: for preoxalated plasma (Tube A) 13-18 sec., for serum-plasma oxalated 30 min. after venipuncture 16-38 sec. (Tube I) and for serum plasma oxalated 60 min. after clotting (Tube II) 33.5-60+ sec.

A significant reduction of *platelets* was noted on the first examination of the blood (21 hours after irradiation began) and thereafter the decrease was progressive. The platelet counts approached zero after the 18th day (Fig. 2).

There was no significant change in *prothrombin concentration* in the blood (Tube A) by the *one-stage method* (Fig. 3). However, in the clotting samples, the one-stage technic was very sensitive to the change following irradiation. Significant changes in prothrombin clotting times were found in coagulating blood 30 minutes after bleeding (Tube I) and 60 minutes after clotting (Tube II). In many cases prothrombin clotting times in Tubes I and II were faster than in Tube A.

When measured by the *2-stage method* normal burro plasma oxalated 30 minutes after venipuncture (Tube I) (Fig. 4) there was an immediate decrease in prothrombin utilization after radiation. Late in the acute whole body irradiation syndrome as much as 90% of residual prothrombin was found.

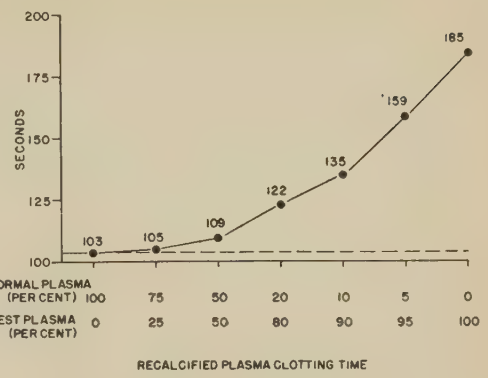

FIG. 5. Mean recalcified plasma clotting times in sec. determined by addition of 0.2 ml of $CaCl_2$ (0.02M) to 0.2 ml of plasma dilutions indicated in abscissa. Broken line represents mean of 5 normal burros. Figures and dots indicate mean values of dilutions of these normal plasmas and the plasmas of 5 individual burros alive 18 days after irradiation.

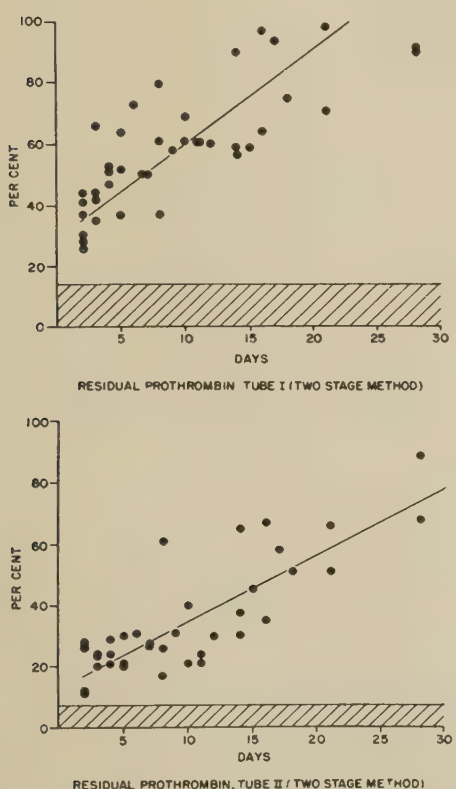

FIG. 4. Residual prothrombin determined by 2-stage method in serum plasma oxalated 30 min. after venipuncture (Tube I) and 60 min. after clotting (Tube II). Shaded areas represent pre-irradiation range (0-14% in Tube I and 0-7% in Tube II), dots represent individual values following irradiation.

Sixty minutes after clotting, the normal burro serum (Tube II) contained 7% or less of the original prothrombin concentration. From 21 hours after irradiation and until death the residual prothrombin concentration in the serum gradually increased. Up to 85% of the total prothrombin remained in the serum one hour after clotting (Fig. 4).

There was a gradual lengthening of the recalcification clotting time (RCT) when the plasma of 5 normal burros was quantitatively diluted with plasma from 5 irradiated burros alive 15 days after radiation (Fig. 5).

Discussion. As in man(12) and other animals(13-16) with few exceptions(17,18) the whole blood clotting time was increased by fatal whole body gamma radiation of burros. In blood of burros having developed a severe clotting deficiency, there were sufficient clotting factors present for a poor venipuncture, introduction of air bubbles or undue agitations to reduce the clotting time materially. Gradation of results, not clearly observed at room temperature in 24 hours were readily detected in one hour at 37°C. Defective clot retraction

due to radiation has been previously reported (13,15,17,18).

There is a relation of platelets to normal whole blood clotting and clot retraction (18-21). The correlation of post irradiation bleeding, clot retraction and prolonged clotting times with thrombocytopenia was reviewed by Cronkite and Brecher(1) and the experimental correction of these defects by platelet transfusion is noted(22). There was no evidence of hypoprothrombinemia in our burros. With a few exceptions, these results generally agree with the findings of other studies(15,16,23).

Defects in prothrombin utilization as demonstrated (Fig. 3, 4) by both one-stage and 2-stage methods are comparable to the results reported by Jackson *et al.*(15), Cronkite *et al.* (24), Penick *et al.*(14), and Ferguson(11). Correction of this defect by platelet transfusion was noted by Dillard, Brecher, and Cronkite(20).

The correlation of the decrease in platelets with prothrombin utilization has been observed experimentally in the blood of dogs(9). Due to different methods of platelet enumerations and the empirical nature of prothrombin determination technics the degree of quantitative correlation of the exact platelet level and the occurrence of defective prothrombin utilization has not been established(9,11,15,24). In some cases there was a deficient prothrombin utilization when burro blood contained 80% of normal platelets.

The results of the 2-stage method always demonstrated a percentage utilization of prothrombin in clotting blood with routinely selected 2-stage reagents. The one-stage method frequently indicated a higher prothrombin activity in serum-plasma samples (Tubes I and II) than in fresh oxalated blood samples (Tube A). This divergence from the 2-stage results was seen in pre-irradiated (normal) blood of burros as in dogs by Langdell *et al.* (10). These workers and Alexander and Landwehr(25) suggest that it is due to a non-prothrombin factor which appeared during clotting and shortened the one-stage "prothrombin" times. With the delayed prothrombin utilization after irradiation there is an opportunity to demonstrate this factor over a longer period during clotting, confirming Ferguson's experience on dogs given Au-198(11).

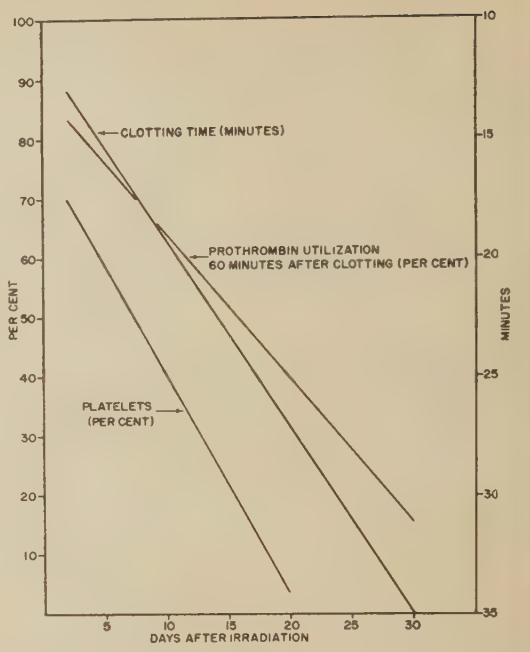

FIG. 6. Trends of reduction of platelets, decrease in prothrombin utilization and prolongation of clotting times from 2nd day after irradiation.

The burro plasma mixture recalcification test data (Fig. 5), unlike those of Ferguson's (11) radio-gold dog experiments, gave evidence of a coagulation defect, but the results must be interpreted as failure to demonstrate any circulating anticoagulant. Lewis and Ferguson (personal communication and(26)) use this test to differentiate between circulating anticoagulant and certain clotting factor deficiencies (*e.g.*, congenital hypoprothrombinemia, hemophilia, PTC lack, etc.). Whether the results of our burro tests are attributable to the thrombocytopenia or to lack of some plasma factor must await further experimental analysis as stressed by Ferguson(11).

Summary and conclusions. 1. Nine burros exposed to a lethal dose of whole body gamma radiation were observed. The burros had symptoms and pathologic changes characteristic of the irradiation syndrome. Four died during the first week and the remaining 5 died before the 30th post irradiation day. These 5 showed hemorrhagic symptoms. 2. There was a retardation of whole blood clot-

ting time. A clotting defect was demonstrated in recalcified oxalated plasmas of burros tested 2 weeks after irradiation. 3. All irradiated burros developed a severe thrombocytopenia and a lessening of clot retraction was noted on the after 4th post irradiation day, with no clot retraction after the 16th day when the platelet count was approaching zero. 4. There was a pronounced diminution of prothrombin utilization rate after irradiation. The first change was noted 22 hours after irradiation or 3 hours after the animals were removed from the exposure field. 5. There was no demonstrable circulating anticoagulant. The possible relation of platelet concentration to the clotting defects is discussed.

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Hibernation of the Lizard, *Anolis carolinensis.* (20114)

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Any attempt to evaluate the comparative biochemistry of hibernation is hindered by the sparsity of information on seasonal changes among reptiles. As part of an extensive study of reptilian biochemistry, analyses of certain metabolic indices were done each month on the lizard, *Anolis carolinensis,* to compare the effect of the seasons on animals obtained from the wild with groups kept under laboratory conditions.

In order to determine whether seasonal temperature changes are primarily responsible for the metabolic variations found, a colony of captive lizards was maintained in a windowless, constant temperature room at the average summer temperature of New Orleans,

28°C. It was divided into 2 groups so that the effect of light could be checked. Since south Louisiana receives 14 hours of sunlight in June and 10 hours in December, these groups were exposed to light for 10 and 14 hours respectively. All animals were acclimatized for at least 3 weeks before being used in any experimental procedure. By the end of 3 months the colony was totally depleted by the frequent sacrifice of animals for analyses and required restocking. Every 4 days the animals were fed meal worms, the larval form of the insect, *Tenebrio molitor*. Since *Anolis* will not drink from a dish but acquires its water from drops on leaves, ferns and ivy were kept in the cages during the investigation and their foliage was generously sprinkled with water every day.

Analytical routine. Fifty freshly caught animals were sacrificed each month along with 50 animals from each of the 2 captive groups. Twenty of these animals from each group were used for blood glucose and liver studies, 20 for estimation of total ether extractives (lipid) and 10 for respiration determinations. All animals were fasted 4 days prior to use since it was found that all traces of food disappear from the digestive tract within this period. The animals used for glucose and liver studies were weighed, decapitated and 0.05 to 0.10 ml of available blood was measured for determination of blood glucose(1). The carcass was opened quickly down the midline, the liver was removed, weighed and immediately dropped into boiling 30% KOH solution for subsequent glycogen analysis(2). Animals used for lipid analysis were dried to constant weight in a vacuum desiccator; the carcass was ground into fine pieces and extracted with ether. The extract was filtered, the ether evaporated and the lipid weighed. Respiration was studied in a closed space respiration chamber which consisted of a liter Erlenmeyer flask fitted by means of a 3 holed rubber stopper to a water manometer, thermometer and a pinch clamp for pressure adjustment. The animals were placed into sacks made of net cloth and conditioned in a darkened incubator at 28°C for 4 hours. The sacks were then suspended from the bottom of the rubber stopper of the manometer unit, and the entire

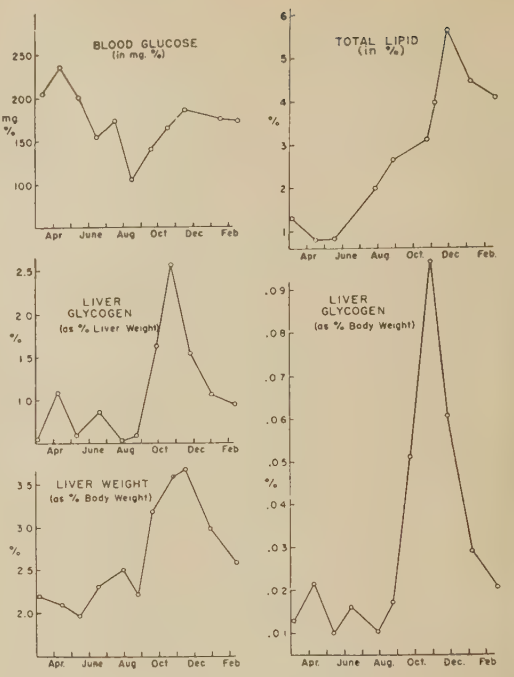

FIG. 1. Seasonal variations *Anolis carolinensis* (nature group).

unit was stoppered into an Erlenmeyer flask which contained 25 ml of standard Ba $(OH)_2$ solution. The respirometer was placed into the darkened incubator. After an hour the manometer was adjusted to atmospheric pressure and temperature and barometer readings were recorded. Forty-four to 52 hours later readings of these same variables and the manometer level were taken, and the $Ba(OH)_2$ was titrated with standard HCl. Oxygen utilization was calculated from the pressure, temperature and manometric changes and CO_2 production from the results of the titration.

Results. A composite picture of the seasonal changes found in animals received fresh from the wild is shown in Fig. 1. Blood glucose varies with the season. Both experimental groups and each sex had practically the same average blood sugar and exhibited the same seasonal periodicity as the controls from the wild.

Liver glycogen increases along with the weight of the liver in the fall. The calculation of liver glycogen as % body weight, therefore, presents a more accurate picture of this increased glycogen storage than does a graph of

% liver glycogen. Although all experimental groups exhibited the same changes as the controls, the livers of the captive animals were always slightly heavier and contained more glycogen. Extrahepatic glycogen also is elevated in the fall and low in the spring. Analyses in September and in February on carcasses remaining after routine liver studies on recently captured animals gave values of 229 and 63 mg/100 g body wt respectively.

In order to test the possibility of keeping the weight of the large livers of the fall animals up by feeding, a group of lizards was placed under dark colony conditions in November and their livers were analyzed in February. Although these animals were given food in the season when wild lizards have a scarcity of food, their livers decreased in weight and glycogen in the same manner as those recently captured.

The ether extractable substances of the dried animal carcass reach a low value in late spring and then increase progressively, reaching a maximum in the month of November after which a gradual decline is noted. Captive animals exhibited the same seasonal periodicity as the control animals from the wild state.

There are no marked seasonal variations in the O_2 utilization or CO_2 production of *Anolis*. All changes from the mean are of the order of 10 to 15%. From March through June O_2 utilization, measured at 28°C, averages 0.198 ± 0.0027* ml/g body wt/hr, whereas during the remainder of the year it averages 0.220 ± 0.0027* ml/g body wt/hr. Although the mean RQ is high (0.914 ± 0.0034*) in all seasons, the lowest individual values recorded occurred in April in animals fresh from the wild.

Discussion. Using standard caloric values for fat and carbohydrate, one can calculate from the data presented that the reserve energy of *Anolis* reaches a peak of about 55 kcal/100 g body wt in the fall and drops to about 10 kcal/100 g body wt in the spring. Only 3% of this reserve energy is due to carbohydrate.

No similar quantitative work on seasonal liver studies on lizards or other reptiles could be found in the literature. Beginning with the work of Athanasiu(3), however, many workers have observed seasonal changes in amphibians. Livers of the frog are larger and have an increased energy content in the fall as compared with other seasons. Blood sugar varies seasonally in the alligator(4) also but remains constant throughout the year in the snakes, *Bathrops jararaca* and *Philodryas sp* (5). If the O_2 consumption of reptiles is measured at the same temperature in different seasons there are no marked changes. *Anolis carolinensis, Alligator mississippiensis*(4), and *Storeria dekayi,* a snake(6) have seasonal differences of less than 15%. In contrast to reptiles the frog, *Rana,* requires 50 to 70% more oxygen in the spring than in the winter (7).

Summary. In late summer and fall, body weight and to a greater extent liver weight increase due to the storage of glycogen and fat. With the increased storage of liver glycogen the blood glucose falls to a low level. In the winter and spring the blood sugar rises to a maximum and the liver glycogen and total lipids fall as the animal uses its body stores. Neither varying the hours of light to which the animals were exposed nor keeping them at a constant temperature throughout the year had any marked effect upon the constituents analyzed.

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* P.E. $= 0.6745 \sqrt{\frac{(v^2)}{n(n-1)}}$.

Isolation and Characterization of Diaminopimelic Acid from Culture Filtrate of an *Escherichia coli* Mutant. (20115)

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Work isolated from acid hydrolysates of a number of bacteria, including *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis*, a ninhydrin-reactive compound that she tentatively characterized as the optically inactive *meso* isomer of α,ϵ-diaminopimelic acid (1). Subsequently Davis showed that certain lysine-requiring auxotrophs of *Escherichia coli* accumulate relatively large amounts of a compound that satisfies the lysine requirement of other auxotrophs. In each of four solvents the accumulated compound had an R_F value determined by either ninhydrin or microbiological response corresponding to diaminopimelic acid and was equally stable to acid and alkali. Certain other *Escherichia coli* mutants that require both diaminopimelic acid and lysine were found to accumulate threonine(2). An enzyme that decarboxylates diaminopimelic acid to yield lysine has been observed by Dewey and Work to occur in many bacteria but to be absent from *Escherichia coli* auxotrophs that require lysine(3).

The difficulties involved in obtaining *Corynebacterium diphtheriae* or *Mycobacterium tuberculosis* cells in quantity and in isolating diaminopimelic acid from such sources are formidable. This report confirms the findings of Davis with respect to the accumulation of relatively large amounts of diaminopimelic acid in culture filtrates of a representative *Escherichia coli* auxotroph and extends the work by describing the details of a convenient procedure by which relatively large amounts of the compound may be readily isolated. In the development of an isolation scheme for obtaining diaminopimelic acid it soon became apparent, as indicated by paper chromatography, that diaminopimelic acid is the main ninhydrin-reactive component of the culture filtrate of an *Escherichia coli* mutant (26-26). It was therefore possible to establish the isolation scheme using the ninhydrin reaction as the only assay procedure. In the final procedure the bacterial centrifugate was treated first with Norit on which all colored products, but no diaminopimelic acid, are adsorbed. The clear filtrate was next passed through a strong anion exchange resin. Diaminopimelic acid above its isoelectric point is retained as an anion by the resin. Cations and other unabsorbed components were washed through the column with a large volume of water. The diaminopimelic acid was eluted from the column with hydrochloric acid. This eluate containing diaminopimelic acid, hydrochloric acid, and other acids was stirred with an excess of a strong anion exchange resin in its bicarbonate form. Anion removal from the eluate was forced to completion because of the instability of carbonic acid, one product of the equilibrium. Under the conditions employed in the removal of the anions, the diaminopimelic acid is not taken above its isoelectric point and no absorption on the resin occurs. The resin filtrate thus obtained was freed of traces of anions and cations by passage through weak anion and cation exchange resins. Diaminopimelic acid was crystallized by the addition of ethanol to a concentrated aqueous solution of the amino acid.*

Diaminopimelic acid obtained by the procedures summarized above was found to have by paper chromatography in 10 different solvent systems R_F values indistinguishable from the corresponding values obtained with authentic synthetic diaminopimelic acid. Even on considerably overloading the papers no more than one ninhydrin-reactive component could be observed. Elementary analysis for carbon, hydrogen, and nitrogen indicated the

* After the isolation scheme described in this paper had been developed we received from Dr. E. Work a submitted manuscript describing the isolation of DAP from an *E. coli* mutant. This method, which differs substantially from ours, had appeared in print (E. Work and R. Denman, *Biochim. et Biophys. Acta*, 1953, v10, 183) at the time proof of the present paper became available. The relative merits of the two procedures remain to be explored.

existence of only small amounts of contamination. Diaminopimelic acid obtained as described above was found to have a small amount of dextrorotation which increased with increasing concentration of hydrochloric acid in the solvent. This behavior according to the rule of Lutz and Jirgensons(4) is characteristic of an amino acid of the L or "natural" configuration. A solubility determination of the amino acid as obtained after one crystallization showed that it is about twice as soluble in water as is the purified diaminopimelic acid obtained by Work from bacterial hydrolysates. Repeated recrystallization of the amino acid from water and ethanol gave a product essentially free of optical rotation and approaching the water insolubility described by Work. Pertinent to these findings with respect to optical rotation and solubility is the corresponding data for cystine the only other naturally-occurring amino acid capable of existing in a *meso* form(5). Meso cystine is considerably less soluble in water than either D- or L-cystine. Mixtures of *meso* and D- or L-cystine can be more soluble than either component alone. It is suggested that in the present instance an analogous situation may prevail in that preparations of diaminopimelic acid with optical activity that show only one spot on paper chromatography but are more soluble than anticipated for the *meso* form may be mixtures of more soluble L (+)- and less soluble *meso*-diaminopimelic acid from which the more insoluble *meso* form is obtained on repeated recrystallization. Obviously the final solution would be facilitated by the availability of all forms of diaminopimelic acid or the discovery of a solvent in which the optically active component of once-crystallized diaminopimelic acid preparations is the more insoluble so that recrystallization to constant rotation could be achieved. The isolated diaminopimelic acid was further characterized by a Van Slyke amino nitrogen determination that showed all nitrogen to be present as α-amino nitrogen. In addition the amino acid was reduced by a 2-step procedure to pimelic acid, demonstrating the absence of branching in the carbon chain. As would be expected, diaminopimelic acid obtained from the representative *Escherichia coli* mutant accumulating the compound is active in satisfying the diaminopimelic acid requirement of another *Escherichia coli* mutant with a nutritional requirement for both diaminopimelic acid and lysine. Diaminopimelic acid from *Escherichia coli* is inactive in satisfying the lysine requirement of *Neurospora crassa*-lysineless (33933), *Streptococcus fecalis* or *Leuconostoc mesenteroides*. Diaminopimelic acid is inactive as a source of threonine for the last two species.

Experimental. Assay method. One hundredth ml of suitably diluted solution or fraction to be examined was evaporated to dryness on filter paper and the ninhydrin reaction carried out as described by Consden *et al.*(6). The qualitative test could be made semi-quantitative by preparing a graded dose series of applications of the solution to the paper and comparing the intensities against those from a similar series prepared from a standard solution of synthetic diaminopimelic acid. The synthetic diaminopimelic acid used in several phases of this study was kindly furnished by Dr. Herschel K. Mitchell.

Microbiological production of the amino acid. Mutant strain 26-26 of *Escherichia coli* that accumulates the amino acid under study was kindly made available by Dr. Bernard D. Davis. Microbiological production of the amino acid was carried out in the medium of Davis and Mingioli(7) modified to contain 0.5% glucose instead of 0.2% and to contain 40 γ/ml of added L (+)-lysine. A flask containing 400 ml of the above medium was seeded from a fresh agar slant of the mutant and after incubation at 30°C for 18 hours with continuous shaking was used to seed 12 liters of medium. After growth at 30°C for 18 hours with continuous agitation and aeration the 12 liter culture was used to inoculate 200 liters of the purified medium contained in an industrial fermenter. The microbiological production was carried out at 30°C for 18 hours with stirring at 50 rpm and aeration with sterile air at a ratio of ¼ volume of air per volume of medium per minute. Following growth of the mutant the cells were centrifuged off with a Sharpless centrifuge and the centrifugate concentrated at 40°C to a volume of 60 liters in a Mojonnier concentrator. This solution was stored in the refrigeration

room and aliquots from it worked up for diaminopimelic acid over a period of several months.

Clarification with Norit. One liter of concentrated bacterial filtrate was shaken with 50 g of Norit A for 30 minutes and then was filtered with suction. The filtrate was clear and colorless.

Absorption on Dowex-1 (OH^-). One liter of norit filtrate was poured on to a column containing 600 g of Dowex-1 resin which had been freshly converted to the OH^- form by liberally washing with 4 liters of 2.5 N NaOH and then with distilled water until the washings were free of alkali. The norit filtrate was passed through the column at a rate of about 10 ml per minute. After application of the norit filtrate the column was washed with distilled water at a flow rate of about 10 ml per minute until the washings were free of alkali. This required about 10 liters of water.

Elution from Dowex-1 (OH^-). The Dowex-1 (OH^-) column containing the bound diaminopimelic acid and anions was eluted with 0.2 N HCl at a flow rate of about 10 ml per minute. Fractions of 500 ml each were collected and by the qualitative ninhydrin test those fractions containing the diaminopimelic acid were located. Those fractions containing most of the diaminopimelic acid were combined.

Anion removal with Dowex-1 (HCO^-_3). The Dowex-1 (OH^-) eluate containing diaminopimelic acid substantially free of cations but containing HCl and acids of other anions contained in the norit-treated bacterial filtrate was freed of these anions by stirring with Dowex-1 (HCO^-_3). The resin was prepared columnwise by washing the commercially available material with sat. $NaHCO_3$ and then distilled water until it was free of alkali. Ordinarily stirring of the hydrochloric acid eluate for 1-2 hours with 200-400 g of Dowex-1 (HCO^-_3) was sufficient to neutralize the anions contained in the solution. When the supernatant solution was alkaline to Congo red paper the resin was filtered off and the filtrate and resin washings were combined and evaporated *in vacuo* to dryness.

Final deionization. The dry residue obtained in the previous step was taken up in 100 ml of H_2O and centrifuged at high speed to remove small amounts of insoluble matter. The centrifugate then was applied to a column containing 100 g of IR-4B freshly prepared in the OH^- form by washing liberally with 2.5 N NaOH and then with distilled water until the washings were free of alkali. The solution to be freed of anions was applied at a flow rate of about 2 ml per minute and was followed by about 300 ml of water until a ninhydrin reaction on the effluent was essentially negative. The combined solution and washings then were applied to a column containing 100 g of IRC-50 freshly prepared in the H^+ form by washing liberally with 1 N HCl and then with distilled water until the washings were free of acid. The solution to be freed of cations was applied at a flow rate of about 2 ml per minute and was followed by about 300 ml of water until a ninhydrin reaction on the effluent was essentially negative.

Crystallization and analysis. The washings from the IR-4B and IRC-50 columns were evaporated *in vacuo* to dryness. The residue was taken up in 30 ml of water and centrifuged at high speed to remove particulate matter. Pure white diaminopimelic acid was then crystallized by the addition of 70 ml of ethanol to the clarified supernatant solution. The cooled suspension was centrifuged and the 70% ethanolic supernatant solution discarded. The product was washed with 7 ml of cold 70% ethanol and then was dried overnight at 105°C. Yield, ca. 260 mg. Analysis:

	C	H	N
Calculated for $C_7H_{14}O_4N_2$	44.20	7.42	14.73
Found (corrected for .62% ash)	43.89	7.48	13.94

A Van Slyke amino nitrogen determination on a separate but equivalent preparation showed 13.63% N. Diaminopimelic acid prepared as described could be recrystallized by solution in water followed by addition of ethanol to a concentration of 70%. One preparation that had been recrystallized five times analyzed as follows:

	C	H	N
Calculated for $C_7H_{14}O_4N_2$	44.20	7.42	14.73
Found (corrected for .44% ash)	44.38	7.95	14.13

Optical activity. The optical activity of once-crystallized diaminopimelic acid with the analysis described above had the rotation summarized as follows:

Solvent	$[\alpha]_{25°}^{D}$ (1.2% solution)
Water	+ 1.2
.05 N HCl	+ 6.3
.2 "	+10.2
2.0 "	+10.5

Other equivalent preparations have shown specific rotations of from +9 to +11.5°. A sample of diaminopimelic acid recrystallized five times from water and ethanol had a rotation of only +1.5° in 2 N HCl.

Water solubility. Thirty mg of once-crystallized diaminopimelic acid was shaken at 23.5°C for 24 hours with 0.4 ml of water in a stoppered tube. The mixture was centrifuged and 0.2 ml of the supernatant was evaporated in a tared weighing dish. A residue of 5.0 mg was obtained, giving a solubility in water of 2.5%. A solubility determination on a sample that has been recrystallized 5 times from water and ethanol gave a solubility, similarly determined except that the temperature was 24.3°, of 1.3%. Work reported that the solubility in water of diaminopimelic acid from a bacterial hydrolysate is 0.92% at 21°C.

Paper chromatography. Three microgram amounts of once-crystallized diaminopimelic acid in 0.01 ml H_2O were spotted near the edge of Whatman No. 1 papers alongside equivalent amounts of synthetic diaminopimelic acid. The paper strips were developed in a number of solvents as indicated below and the position of the isolated and reference diaminopimelic acids was determined by the ninhydrin reaction. In no instance was there any suggestion of more than one ninhydrin-reacting component in the diaminopimelic acid isolated. Separate studies in which 30 μg amounts of diaminopimelic acid were chromatographed with phenol also failed to show the existence of more than one ninhydrin-reactive component even in once-crystallized diaminopimelic acid. RF values are summarized as follows:

Solvent system	RF values: Authentic diaminopimelic acid	RF values: Isolated diaminopimelic acid
Ethanol(7), water(3)	.20	.21
" (1), " (1)	.39	.39
n-Propanol(2), water(1)	.16	.16
n-Butanol (sat. with H_2O)	.01	.01
n-Butanol (sat. with 10% urea)	.01	.01
n-Butanol(4), acetic acid(1), H_2O(5)	.077	.078
Phenol (sat. with H_2O)	.25	.25
n-Butyric acid(9), isovaleric acid(9), H_2O(2)	.061	.062
iso-Amyl alcohol—5% K_2HPO_4	.91	.91
iso-Amyl alcohol—5% KH_2PO_4	.91	.92

Reduction of diaminopimelic acid to pimelic acid. This procedure was carried out stepwise on 107 mg of diaminopimelic acid essentially by the procedure described by Work in which diaminopimelic acid is converted to the corresponding dibromo derivative by reaction with nitrosyl bromide followed by reduction with hydrogen at atmospheric pressure in the presence of platinum black(1). The product obtained after recrystallization three times from benzene and once from water melted at 96.5-98.0°C. An authentic sample of pimelic acid in the same bath melted at 99.0-101.5°C and a mixture of the two melted at 96.5-100.5°C.

Microbiological assays. The activity of diaminopimelic acid as a growth factor for *Escherichia coli* mutant 173-25(2) was determined in the basal medium of Davis and Mingioli(7) according to customary disc-plate assays. Results obtained are summarized as follows:

Conc. of diaminopimelic acid in sol. applied to disc, γ/ml	Zone diameters: Synthetic diaminopimelic acid, mm	Zone diameters: Isolated diaminopimelic acid, mm
100	45	45
50	40	40
25	36	36
12.5	26	27
6.25	18	18
3.125	*	*

* Indistinct.

Determinations of lysine and threonine activity

with *Streptococcus fecalis* R (ATCC 8043) were made according to the procedure of Stokes *et al.*(8) and with *Leuconostoc mesenteroides* (ATCC 8042) according to the procedure of Dunn *et al.*(9). The inability of diaminopimelic acid from *Escherichia coli* to satisfy the lysine requirement of *Neurospora crassa*—lysineless (33933) was determined according to the procedures of Mitchell and Houlahan(10).

Summary. A convenient procedure by which diaminopimelic acid may be readily isolated from the culture filtrate of an *Escherichia coli* mutant is described. The purified diaminopimelic acid resembles that isolated by Work(1) from bacterial hydrolysates with respect to water solubility and absence of significant optical activity. The diaminopimelic acid isolated may be presumed to be the internally compensated *meso* isomer. The possibility that a more water soluble L(+)-diaminopimelic acid may exist in once-crystallized preparations that show only one ninhydrin reactive component was not excluded.

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Effect of Isoniazid on Early Acute Inflammatory Response in Mice. (20116)

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Recent observations on surgical and postmortem lung specimens from patients treated with Isoniazid for tuberculosis revealed extensive healing(1). The granulation tissue in these healing lesions was very vascular and remarkably free from necrosis and inflammation. For this reason it was decided to determine whether Isoniazid had any effect on the host responses apart from its anti-bacterial activity. The local response to inflammation produced by oil of turpentine in mice was investigated.

Experimental procedure. Seventy mice (Swiss Albino) weighing 25-30 g were divided into 2 equivalent groups. One group received 0.25 mg each of Isoniazid (Rimifon) 12 hours prior to subcutaneous injection over the sternal region of 0.025 ml of oil of turpentine. At the time of the oil of turpentine injection, another dose of Isoniazid was administered and thence twice daily until sacrifice. The other group of animals received the oil of turpentine in the same dose and at the same time as the preceding group. Injections of saline were given to this group at the same time that the preceding group received Isoniazid. Fifteen animals in both groups were sacrificed 7 hours and 5 in each group 16, 24, 48 hours and 5 days after oil of turpentine injection. They were autopsied and the area of oil of turpentine injection was excised and prepared for

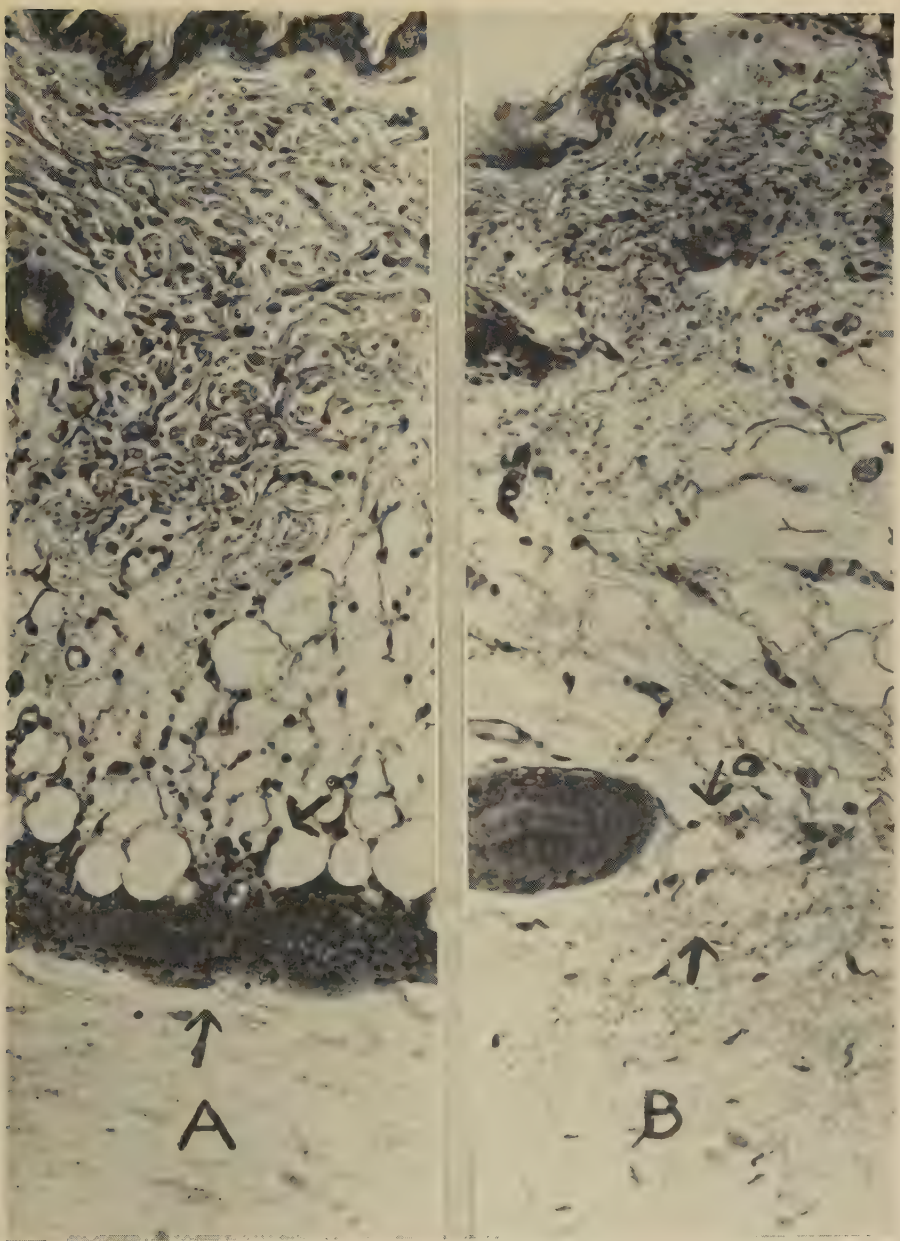

FIG. 1. (A) Photomicrograph (H & E × 350) showing leucocytic zone in control mouse. (B) Photomicrograph (H & E × 350) showing sparse infiltration of leucocytes in Isoniazid treated mouse.

histological examination. The spleens of all animals were weighed and prepared for histological examination. *Another group* of 20 animals were adrenalectomized and divided into 2 groups. One group received 0.25 mg each of Isoniazid 12 hours prior to subcutaneous injection over the sternal region of 0.25 ml of oil of turpentine. At the time of the oil of turpentine injection, another dose of Isoniazid was administered. The other 10 animals received oil of turpentine in the same dose and at the same time, but injections of saline were substituted for Isoniazid. These animals were all killed 7 hours after oil of turpentine

injection and inflammation sites were excised and prepared for histological examinations. The *third group* of 20 mice were wounded on their backs by a special circular punch. These animals were divided into 2 groups and one group received Isoniazid for 5 days in the same dose as the above groups while the other received saline injections. At the end of 5 days the animals were sacrificed. The wounds were excised and prepared for histological examination.

Findings. In the 7-hour post oil of turpentine injection control group of animals (those receiving saline injections) surrounding the site of edema produced by the oil of turpentine was a definite zone of inflammatory cells consisting mainly of polymorphonuclear leucocytes. In this zone there were numerous engorged vessels. The zone of leucocytes was compact and measured anywheres from 5-15 cells in thickness (Fig. 1-A).

In the 7-hour Isoniazid treated group of animals, an area of edema corresponding to the area of oil of turpentine injection was present. However, in this group, the leucocytic zone was distinctly less definite and less cellular (Fig. 1-B) at the margin of the edema. There were in some animals occasional scattered leucocytes, monocytes, and lymphocytes. Engorgement of the vessels was not as pronounced as in the former group. Most animals in this group failed to show a distinct leucocytic zone.

In the 16-hour post turpentine injection animals, the differences between the Isoniazid treated and the control animals was less distinct. No differences were noted in the degree and character of inflammation between the 2 groups at 24, 48 hours, and 5 days. In the adrenalectomized animals, the appearance at the end of 7 hours post turpentine injection was identical to that of the non-adrenalectomized animals.

There was no difference in the spleen size in both groups and no evidence of disintegration of lymphocytes or change in size of the Malpighian bodies.

Examination of the degree of granulation tissue formation in the third group of animals that were wounded and sacrificed at the end of 5 days revealed no differences in the degree of wound healing between the Isoniazid treated animals and the control group.

Discussion. A recent report(2) indicated that irritation of the hind extremities of rats that were treated with Isoniazid showed diminished amount of edema and temperature rise as compared with control animals. The observations reported here support this finding. It seems, therefore, that Isoniazid apart from its specific anti-bacterial activity exerts a non-specific delaying action on the hosts acute inflammatory response. This effect on the inflammatory response occurs only during the early phases and is temporary. This effect apparently is not mediated through the adrenal gland for the findings were essentially the same in the intact animals and in the adrenalectomized animals. There is also apparently no direct effect on the formation of granulation tissue as manifested by the rate of wound healing and its histological appearance. This non-specific inhibition of the acute inflammatory response may be one of the factors responsible for the rather sudden drop in temperature, marked diminution in sputum, and increase in the feeling of well-being in Isoniazid treated patients with tuberculosis.

Summary. 1. Isoniazid temporarily delays the early acute inflammatory response to oil of turpentine in mice. 2. Adrenalectomy does not influence the effect of Isoniazid on early inflammation. 3. Isoniazid has no effect on the rate of wound healing in mice.

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Hydrogenase in Nitrogenase-Deficient Azotobacter Mutants.* (20117)

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Hydrogenase appears to have a unique significance in biological nitrogen fixation because of its occurrence in most organisms capable of fixing molecular nitrogen and its relationship to the nitrogen metabolism of the organism. Lee and Wilson(1) showed that cultures of *Azotobacter* fixing atmospheric N_2 have a greater hydrogenase activity than cultures grown on sources of fixed nitrogen. More recently, Jensen(2) demonstrated that *Azotobacter indicum* grown on ammonium lactate or glutamic acid has no hydrogenase, whereas cultures grown on N_2 possess the enzyme. If hydrogenase is primarily concerned with nitrogen fixation in the azotobacter, strains lacking nitrogenase‡ should also lack hydrogenase or have a markedly decreased level. This report describes the occurrence of hydrogenase in nitrogenase-deficient mutants and reverted strains of the azotobacter.

Methods. The organisms used were *Azotobacter vinelandii* strain O from the culture collection of the University of Wisconsin, *Azotobacter agile* 4.4 obtained from Dr. H. A. Barker, and nitrogenase-deficient strains of these species isolated by penicillin screening (3,4). Isolation of such mutants is achieved by exposing cultures of the wild type growing in a nitrogen-free medium to 500 units of penicillin per ml. The antibiotic has a bactericidal effect on cells which are growing in this medium, *i.e.*, assimilating molecular N_2, but not on spontaneous mutants which are unable to utilize this source of nitrogen. Cultures were grown in Burk's-sucrose medium(5) with or without 300 ppm $(NH_4)_2HPO_4$-nitrogen in 500 ml Erlenmeyer flasks on a rotary shaker (360 rpm) at 30°C. Growth rates (k values) were determined turbidimetrically. Cultures were harvested, washed with M/15 phosphate buffer, pH 8.0, and suspended in 0.2% KCl. Hydrogenase was assayed in a Warburg microrespirometer at 36°C in an atmosphere of hydrogen purified of oxygen by passage through alkaline pyrogallol. The main compartment of the Warburg flask contained 25 μmoles of methylene blue chloride and 300 μmoles of phosphate buffer, pH 8.0. The center well contained 0.2 ml 20% KOH to absorb CO_2, and the sidearm, the cells. Water was added to the main compartment to give a total volume of 3.2 ml. Specific activity of hydrogenase is expressed as $Q_{H_2}(N)$ value, defined as the microliters of hydrogen taken up per hour per mg nitrogen. Nitrogen was determined by the Kjeldahl method.

Results. The effect of age on the level of hydrogenase in *A. agile* and *A. vinelandii* wild types is shown in Fig. 1. Up to 12 hours, the $Q_{H_2}(N)$ of both species is continually lower in ammonia-grown cells than in cells fixing N_2, indicating that ammonium-nitrogen is inhibiting the formation of hydrogenase. The sharp drop in the hydrogenase activity of ammonia-grown cultures after 12 hours probably arises from the increased hydrogen ion concentration of the medium resulting from the selective utilization of the ammonium ion. Cultures of *A. agile* grown on 400 ppm NH_3-N for 24 hours in which the pH was maintained between 6.7 and 7.5 by addition of sterile base showed no such drop in activity after 12 hours, having a specific activity of 9,100 in 24 hours —a 36% inhibition by ammonia since the comparable N_2-grown culture at this age had a $Q_{H_2}(N)$ of 14,300. These results cannot be accounted for by a resumption of nitrogen fixation since the supernatant still contained 164 micrograms NH_3-N per ml.

Mutants of both species of the azotobacter

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‡ For convenience, we shall term cells unable to use molecular N_2 "nitrogenase-deficient," although obviously the block need not occur in the initial step mediated by the enzyme, nitrogenase.

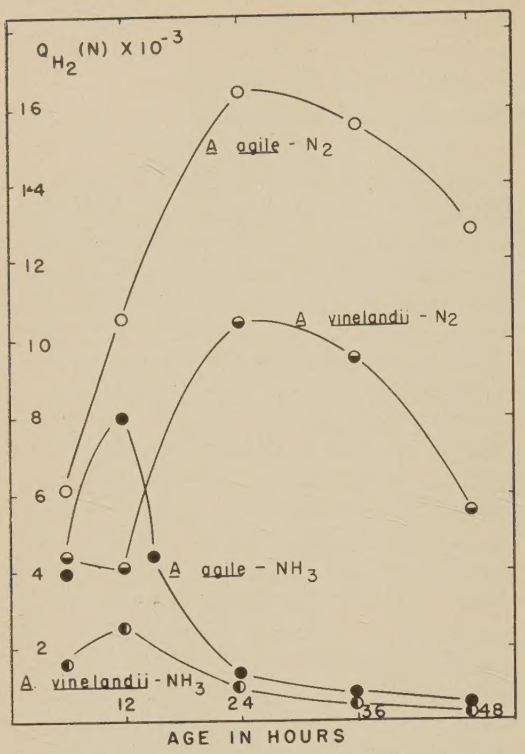

FIG. 1. The time course for production of hydrogenase by the azotobacter. One ml aliquots of 24-hr cultures of the 2 wild types grown in N-free media were inoculated into media either N-free or containing 300 ppm ammonium-N. Cultures were incubated on a rotary shaker (360 rpm) at 30°C and harvested at regular intervals.

unable to use N_2 but capable of utilizing ammonium-nitrogen at varying rates were compared with the wild types with respect to content of hydrogenase. When back mutants which fixed nitrogen arose from the nitrogenase-deficient strains, the hydrogenase activity of these organisms was also assayed to determine the effect of the resumption of fixation on the enzyme content. All cultures were harvested before the pH became sufficiently low to affect the hydrogenase level. One nitrogenase-less mutant of *A. agile* (No. 373) used ammonium-N at a rate approximately equal to that of the wild type, reverting after periods of 3-21 days to nitrogen fixation. Two other types of mutants were isolated and assayed: (a) *A. agile* mutant No. 65 which fixed N_2 at a reduced rate but used ammonium-N at the normal rate; and (b) mutants of *A. agile* and *A. vinelandii* unable to fix and also unable to use ammonium-N at the rate of the wild type. The results of the hydrogenase assays for typical strains are summarized in Table I. All mutants contained hydrogenase, but the specific activity in ammonia-grown cultures was lower than for the same cultures, after back mutation, grown in nitrogen-free media. Repeated transfers of the reverted cultures in nitrogen-free media did not increase the $Q_{H_2}(N)$ value.

Discussion. All non-fixing mutants of the azotobacter had a reduced hydrogenase activity when grown in fixed nitrogen; moreover, those mutants which back-mutated to utilization of N_2 also possessed hydrogenase activity typical of the fixing wild type. In the one mutant (No. 373) which, after reversion to fixation, grew at the same rate as the wild type on N_2, the hydrogenase activity was equal to that of the parent, but it was lower if the growth rate of the mutants (*e.g.*, No. 65) on N-free medium was less than the wild type. These results confirm and extend the previously noted relationship between hydrogenase and the non-symbiotic nitrogen fixing system.

The presence of hydrogenase in the absence of nitrogenase in strains of the azotobacter suggests that hydrogenase functions in other systems of the organism in addition to participation in nitrogen fixation. Since hydrogenase has the lowest reduction potential of any biological system (6), cells of azotobacter may use it in various O/R reactions involving one or more enzymes, coenzymes, or intermediates in the carbon or nitrogen metabolism. For example, the decrease in activity of the serine deaminase of *Escherichia coli* could be prevented by storing in H_2, presumably through the activity of hydrogenase (7).

Study of an azotobacter mutant which does not possess hydrogenase might provide a solution to the nitrogenase-hydrogenase interrelationship. A hydrogenase-deficient strain of *Desulfovibrio desulfuricans* has been isolated by Adams *et al.* (8); although those workers did not determine the ability of their strain to utilize atmospheric nitrogen, Sisler and ZoBell (9) have classified this species among the nitrogen-fixers.

TABLE I. Hydrogenase of Nitrogenase-Deficient Strains of *Azotobacter*.

Organism	Strain	N-free medium		NH_3-N medium	
		k value	$Q_{H_2}(N)$, × 100	k value	$Q_{H_2}(N)$, × 100
A. agile	WT*	.36–.39	13†	.38–.41	8†
	373	.00	—	.34–.36	8.6†
	373‡	.32–.36	16†	—	8.5†
	WT	.39	163	.40	46
	373	.00	—	.34–.36	47
	65	.17	62	.36	51
	54	—	53	.14	17
	55	—	41	.15	19
	59	.00	—	.23	56
A. vinelandii	WT	.33	98	.39	21
	1	.06	38	.25	18
	3	.07	41	.27	19
	19	.07	27	.27	16
	22	.00	—	.26	15

* WT = Wild type.
† Hydrogen acceptor was 150 μmoles of $K_3Fe(CN)_6$ rather than methylene blue.
‡ After reversion to nitrogen fixation.

Summary. Non-fixing mutants of both *Azotobacter agile* and *Azotobacter vinelandii* grown on media containing ammonium-N had a reduced hydrogenase activity similar to that of the wild type. Those mutants which reverted to nitrogen fixation had a level of hydrogenase in N-free media at least double that of the cells grown on fixed nitrogen. The specific activity was equal to that of the wild type on N_2 if the growth rate was normal, but was lower if the growth rate was less than the wild type. The results confirm the close relationship between hydrogenase and nitrogen fixation in the azotobacter but also suggest that hydrogenase in these organisms may have same secondary function as it is present, although to a lesser extent, in mutants unable to use N_2.

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